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**(54) Title: INTERACTION TRAP SYSTEMS FOR DETECTING PROTEIN INTERACTIONS****(57) Abstract**

Disclosed herein is a method of determining whether a first protein is capable of physically interacting with a second protein, involving: (a) providing a host cell which contains (i) a reporter gene operably linked to a protein binding site; (ii) a first fusion gene which expresses a first fusion protein, the first fusion protein including the first protein covalently bonded to a binding moiety which is capable of specifically binding to the protein binding site; and (iii) a second fusion gene which expresses a second fusion protein, the second fusion protein including the second protein covalently bonded to a gene activating moiety and being conformationally-constrained; and (b) measuring expression of the reporter gene as a measure of an interaction between the first and the second proteins. Also disclosed are methods for assaying protein interactions, and identifying antagonists and agonists of protein interactions. Proteins isolated by these methods are also discussed. Finally, populations of eukaryotic cells are disclosed, each cell having a recombinant DNA molecule encoding a conformationally-constrained intracellular peptide.

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- 1 -

INTERACTION TRAP SYSTEMS FOR  
DETECTING PROTEIN INTERACTIONS  
Background of the Invention

5        This invention relates to methods for detecting protein interactions and isolating novel proteins.

Summary of the Invention

In general, the invention features methods for detecting interactions among proteins.

10        Accordingly, in one aspect, the invention features a method of determining whether a first protein is capable of physically interacting with a second protein. The method includes (a) providing a host cell which contains (i) a reporter gene operably linked to a DNA-  
15 binding-protein recognition site; (ii) a first fusion gene which expresses a first fusion protein, the first fusion protein comprising the first protein covalently bonded to a binding moiety which is capable of specifically binding to the DNA-binding-protein  
20 recognition site; and (iii) a second fusion gene which expresses a second fusion protein, the second fusion protein including the second protein covalently bonded to a gene activating moiety and being conformationally-constrained; and (b) measuring expression of the reporter  
25 gene as a measure of an interaction between the first and said second proteins.

Preferably, the second protein is a short peptide of at least 6 amino acids in length and is less than or equal to 60 amino acids in length; includes a randomly generated or intentionally designed peptide sequence; or is conformationally-constrained as a result of covalent bonding to a conformation-constraining protein, e.g., thioredoxin or a thioredoxin-like molecule. Where the second protein is covalently bonded to a conformationally

- 2 -

constraining protein the invention features a polypeptide wherein the second protein is embedded within the conformation-constraining protein to which it is covalently bonded. Where the conformation-constraining 5 protein is thioredoxin, the invention also features an additional method which includes a second protein which is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of the second protein.

10 In another aspect, the invention features a method of detecting an interacting protein in a population of proteins, comprising: (a) providing a host cell which contains (i) a reporter gene operably linked to a DNA-binding-protein recognition site; and (ii) a fusion gene 15 which expresses a fusion protein, the fusion protein including a test protein covalently bonded to a binding moiety which is capable of specifically binding to the DNA-binding-protein recognition site; (b) introducing into the host cell a second fusion gene which expresses a 20 second fusion protein, the second fusion protein including one of said population of proteins covalently bonded to a gene activating moiety and being conformationally-constrained; and (c) measuring expression of the reporter gene. Preferably, the 25 population of proteins includes short peptides of between 1 and 60 amino acids in length.

The invention also features a method of detecting an interacting protein within a population wherein the population of proteins is a set of randomly generated or 30 intentionally designed peptide sequences, or where the population of proteins is conformationally-constrained by covalently bonding to a conformation-constraining protein. Preferably, where the population of proteins is conformationally-constrained by covalent bonding to a 35 conformation-constraining protein, the population of

- 3 -

proteins is embedded within the conformation-constraining protein. The invention further features a method of detecting an interacting protein within a population wherein the conformation-constraining protein is  
5 thioredoxin. Preferably, the population of proteins is inserted into the active site loop of the thioredoxin.

The invention further features a method wherein each of the population of proteins is conformationally-constrained by disulfide bonds between cysteine residues  
10 in the amino-terminus and in the carboxy-terminus of said protein.

In preferred embodiments of various aspects, the host cell is yeast; the DNA binding domain is LexA; and/or the reporter gene is assayed by a color reaction  
15 or by cell viability.

In other embodiments the bait may be Cdk2 or a Ras protein sequence.

In another related aspect, the invention features a method of identifying a candidate interactor. The  
20 method includes (a) providing a reporter gene operably linked to a DNA-binding-protein recognition site; (b) providing a first fusion protein, which includes a first protein covalently bonded to a binding moiety which is capable of specifically binding to the DNA-binding-  
25 protein recognition site; (c) providing a second fusion protein, which includes a second protein covalently bonded to a gene activating moiety and being conformationally-constrained, the second protein being capable of interacting with said first protein; (d)  
30 contacting said candidate interactor with said first protein and/or said second protein; and (e) measuring expression of said reporter gene.

The invention features a method of identifying a candidate interactor wherein the first fusion protein is  
35 provided by providing a first fusion gene which expresses

- 4 -

the first fusion protein and wherein the second fusion protein is provided by providing a second fusion gene which expresses said second fusion protein.

(Alternatively, the reporter gene, the first fusion gene,  
5 and the second fusion gene are included on a single piece  
of DNA.)

The invention also features a method of identifying candidate interactors wherein the first fusion protein and the second fusion protein are  
10 permitted to interact prior to contact with said candidate interactor, and a related method wherein the first fusion protein and the candidate interactor are permitted to interact prior to contact with said second fusion protein.

15 In a preferred embodiment, the candidate interactor is conformationally-constrained. Where the candidate interactor is an antagonist, reporter gene expression is reduced. Where the candidate interactor is an agonist, reporter gene expression is increased. The  
20 candidate interactor is a member selected from the group consisting of proteins, polynucleotides, and small molecules. In addition, a candidate interactor can be encoded by a member of a cDNA or synthetic DNA library. Moreover, the candidate interactor can be a mutated form  
25 of said first fusion protein or said second fusion protein.

In a related aspect, the invention features a population of eukaryotic cells, each cell having a recombinant DNA molecule encoding a conformationally-  
30 constrained intracellular peptide, there being at least 100 different recombinant molecules in the population, each molecule being in at least one cell of said population.

35 Preferably, the intracellular peptides within the population of cells are conformationally-constrained

- 5 -

because they are covalently bonded to a conformation-constraining protein.

In preferred embodiments the intracellular peptide is embedded within the conformation-constraining protein, 5 preferably thioredoxin; the intracellular peptide is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of said second protein; the population of eukaryotic cells are yeast cells; the recombinant DNA 10 molecule further encodes a gene activating moiety covalently bonded to said intracellular peptide; and/or the intracellular peptide physically interacts with a second recombinant protein inside said eukaryotic cells.

In another aspect, the invention features a method 15 of assaying an interaction between a first protein and a second protein. The method includes: (a) providing a reporter gene operably linked to a DNA-binding-protein recognition site; (b) providing a first fusion protein including a first protein covalently bonded to a binding 20 moiety which is capable of specifically binding to the DNA-binding-protein recognition site; (c) providing a second fusion protein including a second protein which is conformationally constrained and covalently bonded to a gene activating moiety; (d) combining the reporter gene, 25 the first fusion protein, and the second fusion protein; and (e) measuring expression of the reporter gene.

The invention further features a method of assaying the interaction between two proteins wherein the first fusion protein is provided by providing a first 30 fusion gene which expresses the first fusion protein and wherein the second fusion protein is provided by providing a second fusion gene which expresses the second fusion protein.

In yet other aspect, the invention features a 35 protein including the sequence Leu-Val-Cys-Lys-Ser-Tyr-

- 6 -

Arg-Leu-Asp-Trp-Glu-Ala-Gly-Ala-Leu-Phe-Arg-Ser-Leu-Phe  
(SEQ ID NO: 1), preferably conformationally-constrained;  
protein including the sequence Met-Val-Val-Ala-Ala-Glu-  
Ala-Val-Arg-Thr-Val-Leu-Leu-Ala-Asp-Gly-Gly-Asp-Val-Thr  
5 (SEQ ID NO: 2); preferably conformationally-constrained;  
a protein including the sequence Pro-Asn-Trp-Pro-His-Gln-  
Leu-Arg-Val-Gly-Arg-Val-Leu-Trp-Glu-Arg-Leu-Ser-Phe-Glu  
(SEQ ID NO: 3), preferably conformationally-constrained;  
a protein including the sequence Ser-Val-Arg-Met-Arg-Tyr-  
10 Gly-Ile-Asp-Ala-Phe-Phe-Asp-Leu-Gly-Gly-Leu-Leu-His-Gly  
(SEQ ID NO: 9), preferably conformationally-constrained;  
a protein including the sequence Glu-Leu-Arg-His-Arg-Leu-  
Gly-Arg-Ala-Leu-Ser-Glu-Asp-Met-Val-Arg-Gly-Leu-Ala-Trp-  
Gly-Pro-Thr-Ser-His-Cys-Ala-Thr-Val-Pro-Gly-Thr-Ser-Asp-  
15 Leu-Trp-Arg-Val-Ile-Arg-Phe-Leu (SEQ ID NO: 10),  
preferably conformationally-constrained; a protein  
including the sequence Tyr-Ser-Phe-Val-His-His-Gly-Phe-  
Phe-Asn-Phe-Arg-Val-Ser-Trp-Arg-Glu-Met-Leu-Ala (SEQ ID  
NO: 11), preferably conformationally-constrained; a  
20 protein including the sequence Gln-Val-Trp-Ser-Leu-Trp-  
Ala-Leu-Gly-Trp-Arg-Trp-Leu-Arg-Arg-Tyr-Gly-Trp-Asn-Met  
(SEQ ID NO: 12), preferably conformationally-constrained;  
a protein including the sequence Trp-Arg-Arg-Met-Glu-Leu-  
Asp-Ala-Glu-Ile-Arg-Trp-Val-Lys-Pro-Ile-Ser-Pro-Leu-Glu  
25 (SEQ ID NO: 13), preferably conformationally-constrained;  
a protein including the sequence Trp-Ala-Glu-Trp-Cys-Gly-  
Pro-Val-Cys-Ala-His-Gly-Ser-Arg-Ser-Leu-Thr-Leu-Leu-Thr-  
Lys-Tyr-His-Val-Ser-Phe-Leu-Gly-Pro-Cys-Lys-Met-Ile-Ala-  
Pro-Ile-Leu-Asp (SEQ ID NO: 17), preferably  
30 conformationally-constrained; a protein including the  
sequence Leu-Val-Cys-Lys-Ser-Tyr-Arg-Leu-Asp-Trp-Glu-Ala-  
Gly-Ala-Leu-Phe-Arg-Ser-Leu-Phe (SEQ ID NO: 18),  
preferably conformationally-constrained; a protein  
including the sequence Tyr-Arg-Trp-Gln-Gln-Gly-Val-Val-  
35 Pro-Ser-Asn-Trp-Ala-Ser-Cys-Ser-Phe-Arg-Cys-Gly (SEQ ID

- 7 -

NO: 19), preferably conformationally-constrained; a protein including the sequence Ser-Ser-Phe-Ser-Leu-Trp-Leu-Leu-Met-Val-Lys-Ser-Ile-Lys-Arg-Ala-Ala-Trp-Glu-Leu-Gly-Pro-Ser-Ser-Ala-Trp-Asn-Thr-Ser-Gly-Trp-Ala-Ser-Leu-5 Ala-Asp-Phe-Tyr (SEQ ID NO: 20) preferably conformationally-constrained; and substantially pure DNA encoding the immediately foregoing proteins.

The invention also includes novel proteins and other candidate interactors identified by the foregoing 10 methods. It will be appreciated that these proteins and candidate interactors may either increase or decrease reporter gene activity and that these changes in activity may be measured using assays described herein or known in the art.

15 As used herein, by "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation, lacZ, amino acid biosynthetic genes, e.g. the yeast LEU2, HIS3, LYS2, TRP1, or URA3 genes, nucleic acid biosynthetic genes, the mammalian 20 chloramphenicol transacetylase (CAT) gene, or any surface antigen gene for which specific antibodies are available. Reporter genes may encode any protein that provides a phenotypic marker, for example, a protein that is necessary for cell growth or a toxic protein leading to 25 cell death, or may encode a protein detectable by a color assay leading to the presence or absence of color (e.g., fluorescent proteins and derivatives thereof). Alternatively, a reporter gene may encode a suppressor tRNA, the expression of which produces a phenotype that 30 can be assayed. A reporter gene according to the invention includes elements (e.g., all promoter elements) necessary for reporter gene function.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to 35 permit gene expression when the appropriate molecules

- 8 -

(e.g., transcriptional activator proteins or proteins which include transcriptional activation domains) are bound to the regulatory sequence(s).

By "covalently bonded" is meant that two domains  
5 are joined by covalent bonds, directly or indirectly.

That is, the "covalently bonded" proteins or protein  
moieties may be immediately contiguous or may be  
separated by stretches of one or more amino acids within  
the same fusion protein.

10 By "providing" is meant introducing the fusion  
proteins into the interaction system sequentially or  
simultaneously, and directly (as proteins) or indirectly  
(as genes encoding those proteins).

By "protein" is meant a sequence of amino acids of  
15 any length, constituting all or a part of a naturally-  
occurring polypeptide or peptide, or constituting a non-  
naturally-occurring polypeptide or peptide (e.g., a  
randomly generated peptide sequence or one of an  
intentionally designed collection of peptide sequences).

20 By a "binding moiety" is meant a stretch of amino  
acids which is capable of directing specific polypeptide  
binding to a particular DNA sequence (i.e., a "DNA-  
binding-protein recognition site").

By "weak gene activating moiety" is meant a  
25 stretch of amino acids which is capable of weakly  
inducing the expression of a gene to whose control region  
it is bound. As used herein, "weakly" is meant below the  
level of activation effected by GAL4 activation region II  
(Ma and Ptashne, Cell 48:847, 1987) and is preferably at  
30 or below the level of activation effected by the B112  
activation domain of Ma and Ptashne (Cell 51:113, 1987).  
Levels of activation may be measured using any downstream  
reporter gene system and comparing, in parallel assays,  
the level of expression stimulated by the GAL4 region II-

- 9 -

polypeptide with the level of expression stimulated by the polypeptide to be tested.

By "altering the expression of the reporter gene" is meant an increase or decrease in the expression of the 5 reporter gene to the extent required for detection of a change in the assay being employed. It will be appreciated that the degree of change will vary depending upon the type of reporter gene construct or reporter gene expression assay being employed.

10 By "conformationally-constrained" is meant a protein that has reduced structural flexibility because its amino and carboxy termini are fixed in space. Preferably, the conformationally-constrained protein is displayed in a structurally rigid manner. Conformational 15 constraint according to the invention may be brought about by exploiting the disulfide-bonding ability of a natural or recombinantly-introduced pair of cysteine residues, one residing at or near the amino-terminal end of the protein of interest and the other at or near the 20 carboxy-terminal end. Alternatively, conformational constraint may be facilitated by embedding the protein of interest within a conformation-constraining protein.

By "conformation-constraining protein" is meant any peptide or polypeptide which is capable of reducing 25 the flexibility of another protein's amino and/or carboxy termini. Preferably, such proteins provide a rigid scaffold or platform for the protein of interest. In addition, such proteins preferably are capable of providing protection from proteolytic degradation and the 30 like, and/or are capable of enhancing solubility.

Examples of conformation-constraining proteins include thioredoxin and other thioredoxin-like proteins, nucleases (e.g., RNase A), proteases (e.g., trypsin), protease inhibitors (e.g., bovine pancreatic trypsin 35 inhibitor), antibodies or structurally-rigid fragments

- 10 -

thereof, and conotoxins. A conformation-constraining peptide can be of any appropriate length and can even be a single amino acid residue.

"Thioredoxin-like proteins" are defined herein as 5 amino acid sequences substantially similar, e.g., having at least 18% homology, with the amino acid sequence of E. coli thioredoxin over an amino acid sequence length of 80 amino acids. Alternatively, a thioredoxin-like DNA sequence is defined herein as a DNA sequence encoding a 10 protein or fragment of a protein characterized by having a three dimensional structure substantially similar to that of human or E. coli thioredoxin, e.g., glutaredoxin and optionally by containing an active-site loop. The DNA sequence of glutaredoxin is an example of a 15 thioredoxin-like DNA sequence which encodes a protein that exhibits such substantial similarity in three-dimensional conformation and contains a Cys....Cys active-site loop. The amino acid sequence of E. coli thioredoxin is described in Eklund et al., EMBO J. 20 3:1443-1449 (1984). The three-dimensional structure of E. coli thioredoxin is depicted in Fig. 2 of Holmgren, J. Biol. Chem. 264:13963-13966 (1989). A DNA sequence encoding the E. coli thioredoxin protein is set forth in Lim et al., J. Bacteriol., 163:311-316 (1985). The three 25 dimensional structure of human thioredoxin is described in Forman-Kay et al., Biochemistry 30:2685-98 (1991). A comparison of the three dimensional structures of E. coli thioredoxin and glutaredoxin is published in Xia, Protein Science 1:310-321 (1992). These four publications are 30 incorporated herein by reference for the purpose of providing information on thioredoxin-like proteins that is known to one of skill in the art. Examples of thioredoxin-like proteins are described herein.

By "candidate interactors" is meant proteins 35 ("candidate interacting proteins") or compounds which

- 11 -

physically interact with a protein of interest; this term also encompasses agonists and antagonists. Agonist interactors are identified as compounds or proteins that have the ability to increase reporter gene expression  
5 mediated by a pair of interacting proteins. Antagonist interactors are identified as compounds or proteins that have the ability to decrease reporter gene expression mediated by a pair of interacting proteins.

"Compounds" include small molecules, generally  
10 under 1000 MW, carbohydrates, polynucleotides, lipids, and the like.

By "test protein" is meant one of a pair of interacting proteins, the other member of the pair generally referred to as a "candidate interactor"  
15 (supra).

By "randomly generated" is meant sequences having no predetermined sequence; this is contrasted with "intentionally designed" sequences which have a DNA or protein sequence or motif determined prior to their  
20 synthesis.

By "mutated" is meant altered in sequence, either by site-directed or random mutagenesis. A mutated form of a protein encompasses point mutations as well as insertions, deletions, or rearrangements.

25 By "intracellular" is meant that the peptide is localized inside the cell, rather than on the cell surface.

By an "activated Ras" is meant any mutated form of Ras which remains bound to GTP for a period of time  
30 longer than that exhibited by the corresponding wild-type form of the protein. By "Ras" is meant any form of Ras protein including, without limitation, N-ras, K-ras, and H-ras.

The interaction trap systems described herein  
35 provide advantages over more conventional methods for

- 12 -

isolating interacting proteins or genes encoding interacting proteins. For example, applicants' systems provide rapid and inexpensive methods having very general utility for identifying and purifying genes encoding a wide range of useful proteins based on the protein's physical interaction with a second polypeptide. This general utility derives in part from the fact that the components of the systems can be readily modified to facilitate detection of protein interactions of widely varying affinity (e.g., by using reporter genes which differ quantitatively in their sensitivity to a protein interaction). The inducible nature of the promoter used to express the interacting proteins also increases the scope of candidate interactors which may be detected since even proteins whose chronic expression is toxic to the host cell may be isolated simply by inducing a short burst of the protein's expression and testing for its ability to interact and stimulate expression of a reporter gene.

If desired, detection of interacting proteins may be accomplished through the use of weak gene activation domain tags. This approach avoids restrictions on the pool of available candidate interacting proteins which may be associated with stronger activation domains (such as GAL4 or VP16); although the mechanism is unclear, such a restriction apparently results from low to moderate levels of host cell toxicity mediated by the strong activation domain.

In addition, the claimed methods make use of conformationally-constrained proteins (i.e., proteins with reduced flexibility due to constraints at their amino and carboxy termini). Conformational constraint may be brought about by embedding the protein of interest within a conformation-constraining protein (i.e., a protein of appropriate length and amino acid composition

- 13 -

to be capable of locking the candidate interacting protein into a particular three-dimensional structure). Examples of conformation-constraining proteins include, but are not limited to, thioredoxin (or other 5 thioredoxin-like proteins), nucleases (e.g., RNase A), proteases (e.g., trypsin), protease inhibitors (e.g., bovine pancreatic trypsin inhibitor), antibodies or structurally-rigid fragments thereof, and conotoxins.

Alternatively, conformational constraint may be 10 accomplished by exploiting the disulfide-bonding ability of a natural or recombinantly-introduced pair of cysteine residues, one residing at the amino terminus of the protein of interest and the other at its carboxy terminus. Such disulfide bonding locks the protein into 15 a rigid and therefore conformationally-constrained loop structure. Disulfide bonds between amino-terminal and carboxy-terminal cysteines may be formed, for example, in the cytoplasm of E. coli trxB mutant strains. Under some conditions disulfide bonds may also form within the 20 cytoplasm and nucleus of higher organisms harboring equivalent mutations, for example, an S. cerevisiae YTR4<sup>-</sup> mutant strain (Furter et al., Nucl Acids Res. 14:6357-6373, 1986; GenBank Accession Number P29509). In addition, the thioredoxin fusions described herein (trx<sup>A</sup> 25 fusions) are amenable to this alternative means of introducing conformational constraint, since the cysteines at the base of peptides inserted within the thioredoxin active-site loop are at a proper distance from one another to form disulfide bonds under 30 appropriate conditions.

Conformationally-constrained proteins as candidate interactors are useful in the invention because they are amenable to tertiary structural analysis, thus facilitating the design of simple organic molecule 35 mimetics with improved pharmacological properties. For

- 14 -

example, because thioredoxin has a known structure, the protein structure between the conformationally constrained regions may be more easily solved using methods such as NMR and X-ray difference analysis.

- 5 Certain conformation-constraining proteins also protect the embedded protein from cellular degradation and/or increase the protein's solubility, and/or otherwise alter the capacity of the candidate interactor to interact.

Once isolated, interacting proteins can also be  
10 analyzed using the interaction trap system, with the signal generated by the interaction being an indication of any change in the proteins' interaction capabilities. In one particular example, an alteration is made (e.g., by standard in vivo or in vitro directed or random  
15 mutagenesis procedures) to one or both of the interacting proteins, and the effect of the alteration(s) is monitored by measuring reporter gene expression. Using this technique, interacting proteins with increased or decreased interaction potential are isolated. Such  
20 proteins are useful as therapeutic molecules (for example, agonists or antagonists) or, as described above, as models for the design of simple organic molecule mimetics.

Protein agonists and antagonists may also be  
25 readily identified and isolated using a variation of the interaction trap system. In particular, once a protein-protein interaction has been recorded, an additional DNA coding for a candidate agonist or antagonist, or preferably, one of a library of potential agonist- or  
30 antagonist-encoding sequences is introduced into the host cell, and reporter gene expression is measured. Alternatively, candidate interactor agonist or antagonist compounds (i.e., including polypeptides as well as non-proteinaceous compounds, e.g., single stranded  
35 polynucleotides) are introduced into an in vivo or in

- 15 -

vitro interaction trap system according to the invention and their ability to effect reporter gene expression is measured. A decrease in reporter gene expression (compared to a control lacking the candidate sequence or 5 compound) indicates an antagonist. Conversely, an increase in reporter gene expression (compared again to a control) indicates an agonist. Interaction agonists and antagonists are useful as therapeutic agents or as models to design simple mimetics; if desired, an agonist or 10 antagonist protein may be conformationally-constrained to provide the advantages described herein. Particular examples of interacting proteins for which antagonists or agonists may be identified include, but are not limited to, the IL-6 receptor-ligand pair, TGF- $\beta$  receptor-ligand 15 pair, IL-1 receptor-ligand pair and other receptor-ligand interactions, protein kinase-substrate pairs, interacting pairs of transcription factors, interacting components of signal transduction pathways (for example, cytoplasmic domains of certain receptors and G-proteins), pairs of 20 interacting proteins involved in cell cycle regulation (for example, p16 and CDK4), and neurotransmitter pairs.

Also included in the present invention are libraries encoding conformationally-constrained proteins. Such libraries (which may include natural as well as 25 synthetic DNA sequence collections) are expressed intracellularly or, optionally, in cell-free systems, and may be used together with any standard genetic selection or screen or with any of a number of interaction trap formats for the identification of interacting proteins, 30 agonist or antagonist proteins, or proteins that endow a cell with any identifiable characteristic, for example, proteins that perturb cell cycle progression. Accordingly, peptide-encoding libraries (either random or 35 designed) can be used in selections or screens which either are or are not transcriptionally-based. These

- 16 -

libraries (which preferably include at least 100 different peptide-encoding species and more preferably include 1000, or 100,000 or greater individual species) may be transformed into any useful prokaryotic or 5 eukaryotic host, with yeast representing the preferred host. Alternatively, such peptide-encoding libraries may be expressed in cell-free systems.

Other features and advantages of the invention will be apparent from the following detailed description 10 thereof, and from the claims.

Brief Description of the Drawings

The drawings are first briefly described.

FIGS. 1A-1C illustrate one interaction trap system according to the invention.

15 FIG. 2 is a diagram of a library vector pJM1.

FIG. 3A is a photograph showing the interaction of peptide aptamers with other proteins.

FIG. 3B illustrates the sequence of exemplary Cdk2 interacting peptides.

20 FIG. 4 illustrates coprecipitation of peptides 3 and 13 by Gst-Cdk2. Lane 1. Gst Beads, extract contains TrxA;

Lane 2. Gst Beads, extract contains TrxA-peptide 3;

Lane 3. Gst Beads, extract contain TrxA-peptide 13;

25 Lane 4. Gst-Cdk2 beads, extract contains TrxA;

Lane 5. Gst-Cdk2 beads, extract contains TrxA-peptide 3; and

Lane 6. Gst-Cdk2, extract contains TrxA-peptide 13.

FIG. 5 illustrates the vector BRM116-H-Ras(G12V).

30 FIG. 6 illustrates the vector pEG202-H-Ras(G12V).

Detailed Description

Applicants have developed a novel interaction trap system for the identification and analysis of conformationally-constrained proteins that either 35 physically interact with a second protein of interest or

- 17 -

that antagonize or agonize such an interaction. In one embodiment, the system involves a eukaryotic host strain (e.g., a yeast strain) which is engineered to produce a protein of therapeutic or diagnostic interest as a fusion 5 protein covalently bonded to a known DNA binding domain; this protein is referred to as a "bait" protein because its purpose in the system is to "catch" useful, but as yet unknown or uncharacterized, interacting polypeptides (termed the "prey"; see below). The eukaryotic host 10 strain also contains one or more "reporter genes," i.e., genes whose transcription is detected in response to a bait-prey interaction. Bait proteins, via their DNA binding domain, bind to their specific DNA recognition site upstream of a reporter gene; reporter transcription 15 is not stimulated, however, because the bait protein lacks an activation domain.

To isolate DNA sequences encoding novel interacting proteins, members of a DNA expression library (e.g., a cDNA or synthetic DNA library, either random or 20 intentionally biased) are introduced into the strain containing the reporter gene and bait protein; each member of the library directs the synthesis of a candidate interacting protein fused to an invariant gene activation domain tag. Those library-encoded proteins 25 that physically interact with the promoter-bound bait protein are referred to as "prey" proteins. Such bound prey proteins (via their activation domain tag) detectably activate the expression of the downstream reporter gene and provide a ready assay for identifying a 30 particular DNA clone encoding an interacting protein of interest. In the instant invention, each candidate prey protein is conformationally-constrained (for example, either by embedding the protein within a conformation-constraining protein or by linking together the protein's 35 amino and carboxy termini). Such a protein is maintained

- 18 -

in a fixed, three-dimensional structure, facilitating mimetic drug design.

An example of one interaction trap system according to the invention is shown in Figures 1 A-C.

5 Figure 1A shows a leucine auxotroph yeast strain containing two reporter genes, LexAop-LEU2 and LexAop-lacZ, and a constitutively expressed bait protein gene. The bait protein (shown as a pentagon) is fused to a DNA binding domain (shown as a circle). The DNA binding 10 protein recognizes and binds a specific DNA-binding-protein recognition site (shown as a solid rectangle) operably-linked to a reporter gene. In Figures 1B and 1C, the cells additionally contain candidate prey proteins (candidate interactors) (shown as an empty 15 rectangle in 1B and an empty hexagon in 1C) fused to an activation domain (shown as a solid square); each prey protein is embedded in a conformation-constraining protein (shown as two solid half circles). Figure 1B shows that if the candidate prey protein does not 20 interact with the transcriptionally-inert LexA-fusion bait protein, the reporter genes are not transcribed; the cell cannot grow into a colony on leu<sup>-</sup> medium, and it is white on Xgal medium because it contains no  $\beta$ -galactosidase activity. Figure 1C shows that, if the 25 candidate prey protein interacts with the bait, both reporter genes are active; the cell forms a colony on leu<sup>-</sup> medium, and cells in that colony have  $\beta$ -galactosidase activity and are blue on Xgal medium. Preferably, in this system, the bait protein (i.e., the 30 protein containing a site-specific DNA binding domain) is transcriptionally inert, and the reporter genes (which are bound by the bait protein) have essentially no basal transcription.

Each component of the system is now described in 35 more detail.

- 19 -

Bait Proteins

The selection host strain depicted in Figures 1 A-C contains a DNA encoding a bait protein fused to a DNA encoding a DNA binding moiety derived from the bacterial LexA protein. The use of a LexA DNA binding domain provides certain advantages. For example, in yeast, the LexA moiety contains no activation function and has no known effect on transcription of yeast genes (Brent and Ptashne, *Nature* 312:612-615, 1984; Brent and Ptashne, *Cell* 43:729-736, 1985). In addition, use of the LexA rather than, for example, the GAL4 DNA-binding domain allows conditional expression of prey proteins in response to galactose induction; this facilitates detection of prey proteins that might be toxic to the host cell if expressed continuously. Finally, the use of a well-defined system, such as LexA, allows knowledge regarding the interaction between LexA and the LexA binding site (i.e., the LexA operator) to be exploited for the purpose of optimizing operator occupancy and/or optimizing the geometry of the bound bait protein to effect maximal gene activation.

Preferably, the bait protein also includes a LexA dimerization domain; this optional domain facilitates efficient LexA dimer formation. Because LexA binds its DNA binding site as a dimer, inclusion of this domain in the bait protein also optimizes the efficiency of operator occupancy (Golemis and Brent, *Mol. Cell Biol.* 12:3006-3014, 1992).

LexA represents a preferred DNA binding domain in the invention. However, any other transcriptionally-inert or essentially transcriptionally-inert DNA binding domain may be used in the interaction trap system; such DNA binding domains are well known and include the DNA binding portions of the proteins ACE1 (CUP1), lambda cI, lac repressor, jun, fos, GCN4, or the Tet repressor. The

- 20 -

GAL4 DNA binding domain represents a slightly less preferred DNA binding moiety for the bait proteins.

Bait proteins may be chosen from any protein of interest and includes proteins of unknown, known, or 5 suspected diagnostic, therapeutic, or pharmacological importance. Preferred bait proteins include oncoproteins (such as myc, particularly the C-terminus of myc, ras, src, fos, and particularly the oligomeric interaction domains of fos) or any other proteins involved in cell 10 cycle regulation (such as kinases, phosphatases, the cytoplasmic portions of membrane-associated receptors). Particular examples of preferred bait proteins include cyclin and cyclin dependent kinases (for example, Cdk2) or receptor-ligand pairs, or neurotransmitter pairs, or 15 pairs of other signalling proteins. In each case, the protein of interest is fused to a known DNA binding domain as generally described herein. Examples are provided below using Cdk2 and Ras baits.

Reporters

20 As shown in Figure 1B, one preferred host strain according to the invention contains two different reporter genes, the *LEU2* gene and the *lacZ* gene, each carrying an upstream binding site for the bait protein. The reporter genes depicted in Figure 1B each include, as 25 an upstream binding site, one or more LexA operators in place of their native Upstream Activation Sequences (UASSs). These reporter genes may be integrated into the chromosome or may be carried on autonomously replicating plasmids (e.g., yeast 2 $\mu$  plasmids).

30 A combination of two such reporters is preferred in the in vivo embodiments of the invention for a number of reasons. First, the LexAop-*LEU2* construction allows cells that contain interacting proteins to select themselves by growth on medium that lacks leucine, 35 facilitating the examination of large numbers of

- 21 -

potential candidate interactor protein-containing cells. Second, the LexAop-lacZ reporter allows LEU<sup>+</sup> cells to be quickly screened to confirm an interaction. And, third, among other technical considerations, the LexAop-LEU2 5 reporter provides an extremely sensitive first selection, while the LexAop-lacZ reporter allows discrimination between proteins of different interaction affinities.

Although the reporter genes described herein represent a preferred embodiment of the invention, other 10 equivalent genes whose expression may be detected or assayed by standard techniques may also be employed in conjunction with, or instead of, the LEU2 and lacZ genes. Generally, such reporter genes encode an enzyme that provides a phenotypic marker, for example, a protein that 15 is necessary for cell growth or a toxic protein leading to cell death, or encoding a protein detectable by a color assay or because its expression leads to the presence or absence of color. Alternatively, the reporter gene may encode a suppressor tRNA whose 20 expression may be assayed, for example, because it suppresses a lethal host cell mutation. Particular examples of other useful genes whose transcription can be detected include amino acid and nucleic acid biosynthetic genes (such as yeast HIS3, URA3, TRP1, and LYS2) GAL1, E. 25 coli galK (which complements the yeast GAL1 gene), and the reporter genes CAT, GUS, fluorescent proteins and derivatives thereof, and any gene encoding a cell surface antigen for which antibodies are available (e.g., CD4). Reporter genes may be assayed by either qualitative or 30 quantitative means to distinguish candidate interactors as agonists or antagonists.

Prey proteins

In the selection described herein, another DNA construction is utilized which encodes a series of 35 candidate interacting proteins (i.e., prey proteins);

- 22 -

each is conformationally-constrained, either by being embedded in a conformation-constraining protein or because the prey protein's amino and carboxy termini are linked (e.g., by disulfide bonding). An exemplary prey 5 protein includes an invariant N-terminal moiety carrying, amino to carboxy terminal, an ATG for protein expression, an optional nuclear localization sequence, a weak activation domain (e.g., the B112 or B42 activation domains of Ma and Ptashne; Cell 51:113, 1987), and an 10 optional epitope tag for rapid immunological detection of fusion protein synthesis. Library sequences, random or intentionally designed synthetic DNA sequences, or sequences encoding conformationally-constrained proteins, may be inserted downstream of this N-terminal fragment to 15 produce fusion genes encoding prey proteins.

Prey proteins other than those described herein are also useful in the invention. For example, cDNAs may be constructed from any mRNA population and inserted into an equivalent expression vector. Such a library of 20 choice may be constructed *de novo* using commercially available kits (e.g., from Stratagene, La Jolla, CA) or using well established preparative procedures (see, e.g., *Current Protocols in Molecular Biology*, New York, John Wiley & Sons, 1987). Alternatively, a number of cDNA 25 libraries (from a number of different organisms) are publicly and commercially available; sources of libraries include, e.g., Clontech (Palo Alto, CA) and Stratagene (La Jolla, CA). It is also noted that prey proteins need not be naturally occurring full-length polypeptides. In 30 preferred embodiments, prey proteins are encoded by synthetic DNA sequences, are the products of randomly generated open reading frames, are open reading frames synthesized with an intentional sequence bias, or are portions thereof. Preferably, such short randomly 35 generated sequences encode peptides between 1 (and

- 23 -

preferably, 6) and 60 amino acids in length. In one particular example, the prey protein includes only an interaction domain; such a domain may be useful as a therapeutic to modulate bait protein activity (i.e., as 5 an antagonist or agonist).

Similarly, any number of activation domains may be used for that portion of the prey molecule; such activation domains are preferably weak activation domains, i.e., weaker than the GAL4 activation region II 10 moiety and preferably no stronger than B112 (as measured, e.g., by a comparison with GAL4 activation region II or B112 in parallel  $\beta$ -galactosidase assays using lacZ reporter genes); such a domain may, however, be weaker than B112. In particular, the extraordinary sensitivity 15 of the LEU2 selection scheme allows even extremely weak activation domains to be utilized in the invention. Examples of other useful weak activation domains include B17, B42, and the amphipathic helix (AH) domains described in Ma and Ptashne (Cell 51:113, 1987), Ruden 20 et al. (Nature 350:426-430, 1991), and Giniger and Ptashne (Nature 330:670, 1987).

The prey proteins, if desired, may include other optional nuclear localization sequences (e.g., those derived from the GAL4 or MAT $\alpha$ 2 genes) or other optional 25 epitope tags (e.g., portions of the c-myc protein or the flag epitope available from Immunex). These sequences optimize the efficiency of the system, but are not required for its operation. In particular, the nuclear localization sequence optimizes the efficiency with which 30 prey molecules reach the nuclear-localized reporter gene construct(s), thus increasing their effective concentration and allowing one to detect weaker protein interactions. The epitope tag merely facilitates a simple immunoassay for fusion protein expression.

- 24 -

Those skilled in the art will also recognize that the above-described reporter gene, DNA binding domain, and gene activation domain components may be derived from any appropriate eukaryotic or prokaryotic source, 5 including yeast, mammalian cell, and prokaryotic cell genomes or cDNAs as well as artificial sequences. Moreover, although yeast represents a preferred host organism for the interaction trap system (for reasons of ease of propagation, genetic manipulation, and large 10 scale screening), other host organisms such as mammalian cells may also be utilized. If a mammalian system is chosen, a preferred reporter gene is the sensitive and easily assayed CAT gene; useful DNA binding domains and gene activation domains may be chosen from those 15 described above (e.g., the LexA DNA binding domain and the B42 or B112 activation domains).

#### Conformation-Constraining Proteins

According to one embodiment of the present invention, the DNA sequence encoding the prey protein is 20 embedded in a DNA sequence encoding a conformation-constraining protein (i.e., a protein that decreases the flexibility of the amino and carboxy termini of the prey protein). Methods for directly linking the amino and carboxy termini of a protein (e.g., through disulfide 25 bonding of appropriately positioned cysteine residues) are described above. As an alternative to this approach, conformation-constraining proteins may be utilized. In general, conformation-constraining proteins act as scaffolds or platforms, which limit the number of 30 possible three dimensional configurations the peptide or protein of interest is free to adopt. Preferred examples of conformation-constraining proteins are thioredoxin or other thioredoxin-like sequences, but many other proteins are also useful for this purpose. Preferably, 35 conformation-constraining proteins are small in size

- 25 -

(generally, less than or equal to 200 amino acids), rigid in structure, of known three dimensional configuration, and are able to accommodate insertions of proteins of interest without undue disruption of their structures. A key feature of such proteins is the availability, on their solvent exposed surfaces, of locations where peptide insertions can be made (e.g., the thioredoxin active-site loop). It is also preferable that conformation-constraining protein producing genes be highly expressible in various prokaryotic and eukaryotic hosts, or in suitable cell-free systems, and that the proteins be soluble and resistant to protease degradation. Examples of conformation-constraining proteins useful in the invention include nucleases (e.g., RNase A), proteases (e.g., trypsin), protease inhibitors (e.g., bovine pancreatic trypsin inhibitor), antibodies or rigid fragments thereof, and conotoxins. This list, however, is not limiting. It is expected that other conformation-constraining proteins having sequences not identified above, or perhaps not yet identified or published, may be useful based upon their structural stability and rigidity.

As mentioned above, one preferred conformation-constraining protein according to the invention is thioredoxin or other thioredoxin-like proteins. As one example of a thioredoxin-like protein useful in this invention, E. coli thioredoxin has the following characteristics. E. coli thioredoxin is a small protein, only 11.7 kD, and can be produced to high levels. The small size and capacity for high level synthesis of the protein contributes to a high intracellular concentration. E. coli thioredoxin is further characterized by a very stable, tight tertiary structure which can facilitate protein purification.

- 26 -

The three dimensional structure of E. coli thioredoxin is known and contains several surface loops, including a distinctive Cys....Cys active-site loop between residues Cys<sub>33</sub> and Cys<sub>36</sub> which protrudes from the body of the protein. This Cys....Cys active-site loop is an identifiable, accessible surface loop region and is not involved in interactions with the rest of the protein which contribute to overall structural stability. It is therefore a good candidate as a site for prey protein insertions. Human thioredoxin, glutaredoxin, and other thioredoxin-like molecules also contain this Cys....Cys active-site loop. Both the amino- and carboxyl-termini of E. coli thioredoxin are on the surface of the protein and are also readily accessible for fusion construction. E. coli thioredoxin is also stable to proteases, stable in heat up to 80°C and stable to low pH.

Other thioredoxin-like proteins encoded by thioredoxin-like DNA sequences useful in this invention share homologous amino acid sequences, and similar physical and structural characteristics. Thus, DNA sequences encoding other thioredoxin-like proteins may be used in place of E. coli thioredoxin according to this invention. For example, the DNA sequence encoding other species' thioredoxin, e.g., human thioredoxin, are suitable. Human thioredoxin has a three-dimensional structure that is virtually superimposable on E. coli's three-dimensional structure, as determined by comparing the NMR structures of the two molecules. Forman-Kay et al., Biochem. 30:2685 (1991). Human thioredoxin also contains an active-site loop structurally and functionally equivalent to the Cys....Cys active-site loop found in the E. coli protein. It can be used in place of or in addition to E. coli thioredoxin in the production of protein and small peptides in accordance with the method of this invention. Insertions into the

- 27 -

human thioredoxin active-site loop and onto the amino terminus may be as well-tolerated as those in E. coli thioredoxin.

Other thioredoxin-like sequences which may be employed in this invention include all or portions of the proteins glutaredoxin and various species' homologs thereof (Holmgren, supra). Although E. coli glutaredoxin and E. coli thioredoxin share less than 20% amino acid homology, the two proteins do have conformational and functional similarities (Eklund et al., EMBO J. 3:1443-1449 (1984)) and glutaredoxin contains an active-site loop structurally and functionally equivalent to the Cys....Cys active-site loop of E. coli thioredoxin. Glutaredoxin is therefore a thioredoxin-like molecule as defined herein.

In addition, the DNA sequence encoding protein disulfide isomerase (PDI), or that portion containing the thioredoxin-like domain, and its various species' homologs thereof (Edman et al., Nature 317:267-270 (1985)) may also be employed as a thioredoxin-like DNA sequence, since a repeated domain of PDI shares >30% homology with E. coli thioredoxin and that repeated domain contains an active-site loop structurally and functionally equivalent to the Cys....Cys active-site loop of E. coli thioredoxin. The two latter publications are incorporated herein by reference for the purpose of providing information on glutaredoxin and PDI which is known and available to one of skill in the art.

Similarly the DNA sequence encoding phosphoinositide-specific phospholipase C (PI-PLC), fragments thereof, and various species' homologs thereof (Bennett et al., Nature, 334:268-270 (1988)) may also be employed in the present invention as a thioredoxin-like sequence based on the amino acid sequence homology with E. coli thioredoxin, or alternatively based on similarity

- 28 -

in three dimensional conformation and the presence of an active-site loop structurally and functionally equivalent to Cys....Cys active-site loop of E. coli thioredoxin. All or a portion of the DNA sequence encoding an 5 endoplasmic reticulum protein, ERp72, or various species homologs thereof are also included as thioredoxin-like DNA sequences for the purposes of this invention (Mazzarella et al., J. Biol. Chem. 265:1094-1101 (1990)) based on amino acid sequence homology, or alternatively 10 based on similarity in three dimensional conformation and the presence of an active-site loop structurally and functionally equivalent to Cys....Cys active-site loop of E. coli thioredoxin. Another thioredoxin-like sequence is a DNA sequence which encodes all or a portion of an 15 adult T-cell leukemia-derived factor (ADF) or other species homologs thereof (Wakasugi et al., Proc. Natl. Acad. Sci. USA, 87:8282-8286 (1990)). ADF is now believed to be human thioredoxin. Similarly, the protein responsible for promoting disulfide bond formation in the 20 periplasm of E. coli, the product of the dsbA gene (Bardwell et al., Cell 67:581-89, 1991) also can be considered a thioredoxin-like sequence. The three latter publications are incorporated herein by reference for the purpose of providing information on PI-PLC, ERp72, ADF, 25 and dsbA which are known and available to one of skill in the art.

It is expected from the definition of thioredoxin-like sequences used above that other sequences not specifically identified above, or perhaps not yet 30 identified or published, may be useful as thioredoxin-like sequences based on their amino acid sequence homology to E. coli thioredoxin or based on having three dimensional structures substantially similar to E. coli or human thioredoxin and having an active-site loop 35 functionally and structurally equivalent to the

- 29 -

Cys....Cys active-site loop of E. coli thioredoxin. One skilled in the art can determine whether a molecule has these latter two characteristics by comparing its three-dimensional structure, as analyzed for example by x-ray crystallography or two-dimensional NMR spectroscopy, with the published three-dimensional structure for E. coli thioredoxin and by analyzing the amino acid sequence of the molecule to determine whether it contains an active-site loop that is structurally and functionally equivalent to the Cys....Cys active-site loop of E. coli thioredoxin. By "substantially similar" in three-dimensional structure or conformation is meant as similar to E. coli thioredoxin as is glutaredoxin. In addition a predictive algorithm has been described which enables the identification of thioredoxin-like proteins via computer-assisted analysis of primary sequence (Ellis *et al.*, Biochemistry 31:4882-91 (1992)). Based on the above description, one of skill in the art will be able to select and identify, or, if desired, modify, a thioredoxin-like DNA sequence for use in this invention without resort to undue experimentation. For example, simple point mutations made to portions of native thioredoxin or native thioredoxin-like sequences which do not effect the structure of the resulting molecule are alternative thioredoxin-like sequences, as are allelic variants of native thioredoxin or native thioredoxin-like sequences.

DNA sequences which hybridize to the sequence for E. coli thioredoxin or its structural homologs under either stringent or relaxed hybridization conditions also encode thioredoxin-like proteins for use in this invention. An example of one such stringent hybridization condition is hybridization at 4X SSC at 65°C, followed by a washing in 0.1X SSC at 65°C for an hour. Alternatively an exemplary stringent hybridization

- 30 -

condition is in 50% formamide, 4X SSC at 42°C. Examples of non-stringent hybridization conditions are 4X SSC at 50°C or hybridization with 30-40% formamide at 42°C. The use of all such thioredoxin-like sequences are believed 5 to be encompassed in this invention.

It may be preferred for a variety of reasons that prey proteins be fused within the active-site loop of thioredoxin or thioredoxin-like molecules. The face of thioredoxin surrounding the active-site loop has evolved, 10 in keeping with the protein's major function as a nonspecific protein disulfide oxido-reductase, to be able to interact with a wide variety of protein surfaces. The active-site loop region is found between segments of strong secondary structure and this provides a rigid 15 platform to which one may tether prey proteins.

A small prey protein inserted into the active-site loop of a thioredoxin-like protein is present in a region of the protein which is not involved in maintaining tertiary structure. Therefore the structure of such a 20 fusion protein is stable. Indeed, E. coli thioredoxin can be cleaved into two fragments at a position close to the active-site loop, and yet the tertiary interactions stabilizing the protein remain.

The active-site loop of E. coli thioredoxin has 25 the sequence NH<sub>2</sub>...Cys<sub>33</sub>-Gly-Pro-Cys<sub>36</sub>...COOH. Fusing a selected prey protein with a thioredoxin-like protein in the active loop portion of the protein constrains the prey at both ends, reducing the degrees of conformational freedom of the prey protein, and consequently reducing 30 the number of alternative structures taken by the prey. The inserted prey protein is bound at each end by cysteine residues, which may form a disulfide linkage to each other as they do in native thioredoxin and further limit the conformational freedom of the inserted prey.

- 31 -

In addition, by being positioned within the active-site loop, the prey protein is placed on the surface of the thioredoxin-like protein, an advantage for use in screening for bioactive protein conformations and 5 other assays. In general, the utility of thioredoxin or other thioredoxin-like proteins is described in McCoy et al., U.S. Pat. No. 5,270,181 and LaVallie et al., Bio/Technology 11:187-193 (1993). These two references are hereby incorporated by reference.

10 There now follows a description of thioredoxin interaction trap systems according to the invention. These examples are designed to illustrate, not limit, the invention.

#### Thioredoxin Interaction Trap System

15 Interaction trap systems utilizing conformationally-constrained proteins have been developed for the detection of protein interactions, for the identification and isolation of proteins participating in such interactions, and for the identification and 20 isolation of agonists and antagonists of such interactions. Exemplary systems are now described.

1. Thioredoxin Interaction Trap with Cdk2 bait

Progression of eukaryotic cells through the cell cycle requires the coordinated action of a number of 25 regulatory proteins that interact with and regulate the activity of Cdks (Sherr, Cell 79:551-555 (1994)). These modulatory proteins include cyclins, which positively regulate Cdk activity, Cyclin Dependent kinase inhibitors (Ckis), and a number of protein kinases and phosphatases, 30 some of which, such as CAK and Cdc25, positively regulate kinase activity, some of which, such as Wee1, inhibit kinase activity, and some of which, such as Cdk1 (Gyuris et al., Cell 75:791-803 (1993)), have effects that are so

- 32 -

far unknown (reviewed in Morgan, *Nature* 374:131-134 (1995)). Cdk2 is thought to be required for higher eukaryotic cells to progress from G1 into S-phase (Fang & Newport, *J. Cell Biol.* 66:731-742 (1991); Pagano et al. 5 *J. Cell Biol.* 121:101-111 (1993); van den Heuvel & Harlow, *Science* 262: 2050-2054 (1993)). Cdk2 kinase activity is positively regulated by Cyclin E and Cyclin A (Koff et al., *Science* 257:1689-1694 (1992); Dulic et al., *Science* 257:1958-1961 (1992); Tsai et al., *Nature* 10 353:174-7 (1991)), negatively regulated by p21, p27 and p57 (Harper et al., *Cell* 75:805-816 (1993); Polyak et al., *Genes Dev.* 8:9-22 (1994); Toyoshima & Hunter, *Cell* 78:67-74 (1994); Matsuoka et al. *Genes Dev.* 9:660-662 (1995); Lee et al., *Genes Dev.* 9:639-649 (1995)); in 15 addition, Cdk2 complexes with Cdil at the G1 to S transition (Gyuris et al., *Cell* 75:791-803 (1993)). Here we describe the use of a yeast two-hybrid system to select molecules which recognize Cdk2 from combinatorial libraries.

20 A prey vector is constructed containing the E. coli thioredoxin gene (trxA). pJG 4-4 (Gyuris et al., *Cell* 75:791, 1993) is used as the vector backbone and cut with EcoRI and XhoI. A DNA fragment encoding the B112 transcription activation domain is obtained by PCR 25 amplification of plasmid LexA-B112 (Doug Ruden, Ph.D. thesis, Harvard University, 1992) and cut with MunI and NdeI. The E. coli trxA gene is excised from the vector pALTRXA-781 (U.S. Pat. No. 5,292,646; InVitrogen Corp., San Diego, CA) by digestion with NdeI and SalI. The trxA 30 and B112 fragments are then ligated by standard techniques into the EcoRI/XhoI-cut pJG 4-4 backbone, forming pYENAeTRX. This vector encodes a fusion protein comprising the SV40 nuclear localization domain, the B112 transcription activation domain, an hemagglutinin epitope 35 tag, and E. coli thioredoxin (Fig. 2A).

- 33 -

Peptide libraries are constructed as follows. The DNA oligomer 5' GACTGACTGGTCCG(NNK)<sub>20</sub>GGTCCTCAGTCAGTCAG 3' (with N = A, C, G, T and K = G, T) (SEQ ID NO: 4) is synthesized and annealed to the second oligomer (5' 5 CTGACTGACTGAGGACC 3') (SEQ ID NO: 5) in order to form double stranded DNA at the 3' end of the first oligomer. The second strand is enzymatically completed using Klenow enzyme, priming synthesis with the second oligomer. The product is cleaved with Avall, and inserted into RsrII 10 cut pYENAeTRX. After ligation, the construct is used to transform E. coli by standard methods (Ausubel et al., supra). The library contained 2.9 x 10<sup>9</sup> members, of which more than 10<sup>9</sup> directs the synthesis of peptides.

To screen for interacting peptides, 20 µg of the 15 library is used to transform the yeast strain EGY48 (Mata his3 leu2::2Lexop-LEU2 ura3 trp1 LYS2; Gyuris et al., supra). This strain also contains the reporter plasmid pSH 18-34, a pLR1Δ1 derivative, containing the yeast 2µ replication origin, the URA3 gene, and a GAL1-lacZ 20 reporter gene with the GAL1 upstream regulatory elements replaced with 4 colE1 LexA operators (West et al., Mol. Cell Biol. 4:2467, 1984; Ebina et al., J. Biol. Chem. 258:13258, 1983; Hanes and Brent, Cell 57:1275, 1989), as well as the bait vector pLexA202-Cdk2 (Cdk2 encodes the 25 human cyclin dependent kinase 2, an essential cell cycle enzyme) (Gyuris et al., supra; Tsai et al., Oncogene 8:1593, 1993). About 2.5 x 10<sup>6</sup> transformants are obtained and pooled. The first selection step, growth on leucine-deficient medium after induction with 2% 30 galactose/1% raffinose (Gyuris et al., supra; Guthrie and Fink, Guide to Yeast Genetics and Molecular Biology, Vol. 194, 1991), is performed with an 8-fold redundancy (20 x 10<sup>6</sup> cfu) of the library in yeast, and about 900 colonies are obtained after growth at 30°C for 5 days. The 300 35 largest colonies are streak purified and tested for the

- 34 -

galactose-dependent expression of the *LEU2* gene product and of  $\beta$ -galactosidase (encoded by pSH 18-34), the latter giving rise to blue yeast colonies in the presence of Xgal in the medium (Ausubel et al., *supra*). Thirty-three 5 colonies fulfill these requirements which, after sequencing, include 14 different clones all of which bind specifically to a LexA-Cdk2 bait, but not to LexA or to a LexA-Cdk3 bait (Finley et al., Proc. Natl. Acad. Sci., 1994). The strength of binding is judged according to 10 the intensity of the blue color formed by a colony of the yeast that contains each different interactor. By this means, each interactor is classified as a strong, medium, or weak binder, which is normalized to the amount of blue color caused by the various naturally-occurring partner 15 proteins of Cdk2 in side by side mating interaction assays. An example of the peptide sequence of one representative of each class is given here:

Strong binder: peptide 3 (SEQ ID NO: 6)

-Gly<sub>34</sub>-Pro<sub>35</sub>-Leu-Val-Cys-Lys-Ser-Tyr-Arg-Leu-Asp-Trp-  
20 Glu-Ala-Gly-Ala-Leu-Phe-Arg-Ser-Leu-Phe-Gly<sub>34</sub>-Pro<sub>35</sub>-

Medium binder: peptide 2 (SEQ ID NO: 7)

-Gly<sub>34</sub>-Pro<sub>35</sub>-Met-Val-Val-Ala-Ala-Glu-Ala-Val-Arg-Thr-  
Val-Leu-Leu-Ala-Asp-Gly-Gly-Asp-Val-Thr-Gly<sub>34</sub>-Pro<sub>35</sub>-

Weak binder: peptide 6 (SEQ ID NO: 8)

25 -Gly<sub>34</sub>-Pro<sub>35</sub>-Pro-Asn-Trp-Pro-His-Gln-Leu-Arg-Val-Gly-  
Arg-Val-Leu-Trp-Glu-Arg-Leu-Ser-Phe-Glu-Gly<sub>34</sub>-Pro<sub>35</sub>-

Control peptides which do not bind detectably are: c4:  
Arg-Arg-Ala-Ser-Val-Cys-Gly-Pro-Leu-Leu-Ser-Lys-Arg-Gly-  
Tyr-Gly Pro-Pro-Phe-Tyr-Leu-Ala-Gly-Met-Thr-Ala-Pro-Glu-  
30 Gly-Pro-Cys (SEQ ID NO: 14) and c: Arg-Arg-Ala-Ser-Val-  
Cys-Gly-Pro-Leu-His-Tyr-Trp-Gly-Leu-Gly-Gly-Phe-Val-Asp-

- 35 -

Leu-Trp-Gln-Glu-Thr-Thr-Gly-Val-Gly-Pro-Cys (SEQ ID NO: 15).

Figure 3A shows that 5 of the peptides reacted strongly with the LexA-Cdk2 bait but not with a large number of unrelated proteins. None of the Cdk2 aptamers interacted with CDC28 or Cdc2, which are both 65% identical to Cdk2. However, 2 of the 5 Cdk2 interactors also interacted with human Cdk3, and 1 of the 5 also interacted with Drosophila Cdc2c, suggesting that these peptides recognize determinants common to these proteins.

Both theoretical considerations and calibration experiments with lambda repressor's C terminus suggest that transcription of the pSH18-34 reporter in EGY48 can be activated by protein interactions with Kds as weak as  $10^{-6}$ M. The fact that peptides 3 and 13 direct robust transcription of the this LexAop-lacZ reporter is consistent with the idea that they may interact significantly more tightly. The sequence of these peptides is shown in Figure 3B. Two of the peptides are longer than unit length; both are apparently artifacts of the *in vitro* manipulations used to construct the library. No peptide showed significant sequence similarity to known proteins, and none showed more than random similarity to any other, suggesting that we have not exhausted the peptide motifs capable of recognizing Cdk2.

To confirm the specificity of the Cdk2 interaction, we immobilized a Gst-Cdk2 fusion protein on glutathione sepharose beads, and used these beads to specifically precipitate two bacterially expressed peptide aptamers (Fig. 4). Gst-Cdk2 was expressed in *E. coli* and purified on glutathione sepharose as described (Lee et al., *Nature* 374: 91-94 (1995)). Peptides 3 and 13 were made as follows: fragments that directed the synthesis of peptides 3 and 13 were made by PCR amplification of the insert encoded by the corresponding

- 36 -

library plasmid and introduced into pAL-TrxA (LaVallie et al., Bio/Technology 11:187-193 (1993)). Fusion proteins were expressed and lysed in a French pressure cell as described (LaVallie et al., BIO/Technology 11, 187-193 5 (1993)). Coprecipitation with Gst-Sepharose beads was done as described (Lee et al., Nature 374, 91-94 (1995)), and samples were run on a 15% SDS polyacrylamide gel and transferred to nylon membranes. TrxA-containing fusion proteins were visualized by probing the membranes with an 10 anti-TrxA antibody, and developing the immobilized antibody with peroxidase-coupled anti-rabbit IgG antibody ECL reagents according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

These experiments demonstrate that the 15 interactions between Cdk2 and the peptide aptamers can be observed *in vitro*, and is thus independent of any bridge proteins native to yeast. Once identified, these peptides may be used in competition experiments.

The ability to select TrxA-peptides that interact 20 specifically with designated intracellular baits allows for the creation of other classes of intracellular reagents. For example, appropriately derivitized TrxA-peptide fusions may allow the creation of antagonists or agonists (as described above). Alternatively, peptide 25 fusions allow for the creation of homodimeric or heterodimeric "matchmakers," which force the interaction of particular protein pairs. In one particular example, two proteins are forced together by utilizing a leucine zipper sequence attached to a conformation-constraining 30 protein containing a candidate interaction peptide. This protein can bind to both members of a protein pair of interest and direct their interaction. Alternatively, the "matchmaker" may include two different sequences, one having affinity for a first polypeptide and the second 35 having affinity for the second polypeptide; again, the

- 37 -

result is directed interaction between the first and second polypeptides. Another practical application for the peptide fusions described herein is the creation of "destroyers," which target a bound protein for 5 destruction by host proteases. In an example of the destroyer application, a protease is fused to one component of an interacting pair and that component is allowed to interact with the target to be destroyed (e.g., a protease substrate). By this method, the 10 protease is delivered to its desired site of action and its proteolytic potential effectively enhanced. Yet another application of the fusion proteins described herein are as "conformational stabilizers," which induce target proteins to favor a particular conformation or 15 stabilize that conformation. In one particular example, the ras protein has one conformation that signals a cell to divide and another conformation that signals a cell not to divide. By selecting a peptide or protein that stabilizes the desired conformation, one can influence 20 whether a cell will divide. Other proteins that undergo conformational changes which increase or decrease activity can also be bound to an appropriate "conformational stabilizer" to influence the property of the desired protein.

25 2. Functional Inhibition of Cdk2

To determine whether Cdk2 interacting peptides might inhibit Cdk2 function *in vivo*, we took advantage of the fact that human Cdk2 can complement temperature sensitive alleles of Cdc28 (Elledge and Spottswood, EMBO 30 10:2653-2659, 1991; Ninomiya et al., PNAS 88:9006-9010, 1991; Meyerson et al., EMBO 11:2909-2917, 1992). Peptide 13 inhibits the plating efficiency of a Cdk2-dependent yeast. A strain carrying the temperature sensitive cdc28-1N mutation can form colonies at high temperature

- 38 -

if it carries a plasmid that expresses Cdk2. At the restrictive temperature, compared to the plating efficiency of yeast expressing control peptides, expression of peptide 13 diminishes the plating 5 efficiency of this strain by 10-fold. Both peptide 3 and 13 have similar effects on the plating efficiency at 37°C of a Cdk2(+) strain that carries the cdc28-13ts allele.

Expression of peptide 13 slows the doubling time of a Cdk2(+), cdc28ts-1N strain by a factor of 50%. 10 Microscopic examination of strains expressing the peptide revealed that a high proportion of these cells had an elongated morphology characteristic of cdc28-1N cells at the restrictive temperature, whereas cells expressing a control peptide had a more normal morphology.

15 Peptide 13 does not affect the growth of a cdc28-1Nts strain at high temperature when the defect is complemented by a plasmid expressing wild-type Cdc28 product, and has no effect on yeast at the permissive temperature. While we do not intend to be bound by any 20 particular theory, it appears that this peptide blocks yeast cell cycle progression by binding to some face of the Cdk2 molecule and inhibiting its function and thereby interfering with its ability to interact with cyclins, other partners, or with substrates.

25 3. Thioredoxin Interaction Trap with OncoRas Bait

The ras proteins are essential for many signal transduction pathways and regulate numerous physiological functions including cell proliferation. The ras genes were first identified from the genome of Harvey and 30 Kirsten sarcoma virus. The three types of mammalian ras genes (N-, K-ras, and H-ras) encode highly conserved membrane-bound guanine nucleotide binding proteins with a molecular mass of 21 kDa, which cycle between the active (GTP-bound) form and the inactive (GDP-bound) form.

- 39 -

In normal cells, the active form of Ras is short-lived, as its intrinsic GTPase activity rapidly converts the bound-GTP to GDP. The GTPase activity is stimulated 10<sup>5</sup>-fold by GTPase-activating proteins (GAPs). GTP-bound 5 Ras interact with GAP, c-Raf, neurofibromatosis type 1 (NF-1) and Ral guanine nucleotide dissociation stimulator (RalGDS).

Mutationally-activated RAS proteins are found in about 30% of human tumor cells and have greatly decreased 10 GTPase activity which can not be stimulated by GAPs. The majority of mutations studied thus far are due to a point mutation at either residue Gly-12 or residue Gln-61 of Ras. These Ras mutants remain in the active form and interact with the downstream effectors to result in 15 tumorigenesis. It has been shown that there are significant conformational differences between GTP-bound forms of wild-type and oncogenic RAS proteins. Such conformational differences are likely causes for malignant transformation induced by oncogenic ras 20 proteins.

Such mutationally-activated conformational changes in GTP-bound H-ras mutants provide targets for members of a conformationally constrained random peptide library. In the present example, the library is a conformationally 25 constrained thioredoxin peptide library, as described above. Library members, which interact with oncogenic Ras have been identified using a variation of the interaction trap technology provided above. The oncogenic Ras peptide aptamers isolated may be assayed 30 for their ability to disrupt the interaction of oncogenic Ras with known effectors and to inhibit cellular transformation.

We have used well-characterized oncogenic H-ras(G12V) for isolation and characterization of its 35 peptide aptamers. Peptide aptamers for other oncogenes

- 40 -

can be isolated using adaptations of this protocol as provided herein.

*Bait Construction*

Construction of LexA-Ras(G12V)/pEG202:H-Ras(G12V)

5 DNA was performed by digesting BTM116-H-Ras(G12V) (Fig. 5) with BamHI and SalI. H-Ras(G12V) DNA was ligated with pEG202 backbone digested with BamHI and SalI. The resulting plasmid was called pEG202-H-Ras(G12V) (or V6) (Fig. 6).

10 *Screening for H-Ras(G12V) peptide aptamers*

pEG202-H-Ras(G12V) (V6) was transformed into the EGY48 strain according to a standard yeast transformation protocol; in particular, the protocol provided by Zymo Research (Orange County, CA) was used here. EGY48 was 15 grown in YPD medium to OD<sub>600</sub>=0.2-0.7. Cells were pelleted at 500 X g for 4 min. and resuspended in 10 ml of EZ1 solution (Zymo Research). The cells were then pelleted by centrifugation and resuspended in 1 ml of EZ2 (Zymo Research). Aliquots of competent cells (50 µl) were 20 stored in a -70°C freezer.

An aliquot of competent cells was mixed with 0.1 µg of LexA-H-Ras(G12V)/pEG202 and 500 µl of EZ3 solution (Zymo Research). The mixture was incubated at 30°C for 30 min. and plated onto a yeast medium lacking histidine 25 and uracil. One colony was picked and inoculated into 100 ml of glucose Ura<sup>-</sup>His<sup>-</sup> medium at 30°C with shaking (150 rpm) until the OD<sub>600</sub> measurement was 0.96. The culture was centrifuged at 2000g for 5 min and cell pellets were resuspended in 5 ml of sterile LiOAc/TE.

30 The cells were again centrifuged as above and resuspended in 0.5 ml of sterile LiOAc/TE.

Aliquots (50 µl) of the cells were then incubated at 30°C for 30 min. with 1 µg of thioredoxin peptide library DNA, 70 µg of salmon sperm DNA, and 300 µl of 35 sterile 40% PEG 4000 in LiOAc/TE. The mixtures were

- 41 -

heat-shocked at 42°C for 15 min. Each aliquot was plated onto a 24 cm x 24 cm plate containing glucose Ura<sup>-</sup>His<sup>-</sup>Trp<sup>-</sup> medium and was incubated at 30°C for two days. The transforming efficiency typically ranged from 50,000 to 5 100,000 colony forming units per µg of library DNA.

A total of 1.5 million transformants were obtained and were plated onto the selection medium of galactose/raffinose Leu<sup>-</sup>Ura<sup>-</sup>His<sup>-</sup>Trp<sup>-</sup>. Of the 338 colonies formed, among them 50 were randomly picked and inoculated 10 into 5 ml of glucose Leu<sup>-</sup>Ura<sup>-</sup>His<sup>-</sup>Trp<sup>-</sup> medium for preparation of yeast plasmid DNA. A half ml of each yeast culture was mixed with an equal volume of acid-washed sand and phenol/chloroform/isoamyl alcohol (24:24:1), and vortexed in a vortexer for 2 min. The 15 mixture was then centrifuged for 15 min., and the supernatant was precipitated with ethanol. DNA pellets were resuspended in 50 µl of TE.

One µl of each sample was used to transform *E. coli* KC8 cells by electroporation. Bacterial 20 transformants were selected on minimal agar supplemented with uracil, leucine, histidine, and ampicillin. Each type transformant resulted in final isolation of plasmid which a leucine marker, which carries a DNA fragment encoding thioredoxin-peptide fusion protein.

Sequence determination of the 50 isolates was 25 carried out according to the directions of the fmolDNA™ sequencing systems (Promega, Madison, WI) using primer 5'-GACGGGGCGATCCTCGTCG-3' (SEQ ID NO:16). Nine out of 50 isolates (referred to as #4, #18, #39, #41, #22, #24, 30 #30, #31, #46) contained unique peptide encoding sequences, as determined by electrophoresis of the dT/ddT termination reaction. Among them, the predicted peptide aptamer sequence of #39 is as follows:  
Trp-Ala-Glu-Trp-Cys-Gly-Pro-Val-Cys-Ala-His-Gly-Ser-Arg-  
35 Ser-Leu-Thr-Leu-Leu-Thr-Lys-Tyr-His-Val-Ser-Phe-Leu-Gly-

- 42 -

Pro-Cys-Lys-Met-Ile-Ala-Pro-Ile-Leu-Asp (SEQ ID NO:17). From our results, it appears that approximately 60 unique H-Ras(G12V) peptide aptamers (338 x 9/50) were isolated in the first round of screening.

5

Other Embodiments

As described above, the invention features a method for detecting and analyzing protein-protein interactions. Typically, in the above experiments, the bait protein is fused to the DNA binding domain, and the prey protein (in association with the conformation-constraining protein) is fused to the gene activation domain. The invention, however, is readily adapted to other formats. For example, the invention also includes a "reverse" interaction trap in which the bait protein is fused to a gene activation domain, and the prey protein (in association with a conformation-constraining protein) is fused to the DNA binding domain. Again, an interaction between the bait and prey proteins results in activation of reporter gene expression. Such a "reverse" interaction trap system, however, depends upon the use of prey proteins which do not themselves activate downstream gene expression.

The protein interaction assays described herein can also be accomplished in a cell-free, in vitro system. Such a system begins with a DNA construct including a reporter gene operably linked to a DNA-binding-protein recognition site (e.g., a LexA binding site). To this DNA is added a bait protein (e.g., any of the bait proteins described herein bound to a LexA DNA binding domain) and a prey protein (e.g., one of a library of conformationally-constrained candidate interactor prey proteins bound to a gene activation domain). Interaction between the bait and prey protein is assayed by measuring the reporter gene product, either as an RNA product, as

- 43 -

an in vitro translated protein product, or by some enzymatic activity of the translated reporter gene product. This in vitro system may also be used to identify agonists or antagonists, simply by adding to a 5 known pair of interacting proteins (in the above described system) a candidate agonist or antagonist interactor and assaying for an increase or decrease (respectively) in reporter gene expression, as compared to a control reaction lacking the candidate compound or 10 protein. To facilitate large scale screening, candidate prey proteins or candidate agonists or antagonists may be initially tested in pools, for example, of ten or twenty candidate compounds or proteins. From pools demonstrating a positive result, the particular 15 interacting protein or agonist or antagonist is then identified by individually assaying the components of the pool. Such in vitro systems are amenable to robotic automation or to the production of kits. Kits including the components of any of the interaction trap systems 20 described herein are also included in the invention.

The components (e.g., the various fusion proteins or DNA therefor) of any of the in vivo or in vitro systems of the invention may be provided sequentially or simultaneously depending on the desired experimental 25 design.

- 44 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: INTERACTION TRAP SYSTEMS FOR  
DETECTING PROTEIN INTERACTIONS

(iii) NUMBER OF SEQUENCES: 20

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version  
#1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20  
(B) TYPE: amino acid  
(C) STRANDEDNESS:

- 45 -

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Val Cys Lys Ser Tyr Arg Leu Asp Trp Glu Ala Gly Ala Leu Phe  
1 5 10 15  
Arg Ser Leu Phe  
20

(2) INFORMATION FOR SEQUENCE ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Val Ala Ala Glu Ala Val Arg Thr Val Leu Leu Ala Asp Gly  
1 5 10 15  
Gly Asp Val Thr  
20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Asn Trp Pro His Gln Leu Arg Val Gly Arg Val Leu Trp Glu Arg  
1 5 10 15  
Leu Ser Phe Glu  
20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 91  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ix) FEATURE:

(D) OTHER INFORMATION: N is A or T or G or C; K is G or T.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GACTGACTGG TCCGNNKNNK NNKNNKNNKN NKNNKNNKNN KNNKNNKNNK NNKNNKNNKN 60

- 46 -

NKNNKNNKNN KNNKGTCCT CAGTCAGTCA G

31

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGACTGACT GAGGACC

17

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly	Pro	Leu	Val	Cys	Lys	Ser	Tyr	Arg	Leu	Asp	Trp	Glu	Ala	Gly	Ala
1				5					10					15	
Leu	Phe	Arg	Ser	Leu	Phe	Gly	Pro								
				20											

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly	Pro	Met	Val	Val	Ala	Ala	Glu	Ala	Val	Arg	Thr	Val	Leu	Leu	Ala
1				5					10				15		
Asp	Gly	Gly	Asp	Val	Thr	Gly	Pro								
				20											

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

- 47 -

Gly Pro Pro Asn Trp Pro His Gln Leu Arg Val Gly Arg Val Leu Trp  
1 5 10 15  
Glu Arg Leu Ser Phe Glu Gly Pro  
20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Val Arg Met Arg Tyr Gly Ile Asp Ala Phe Phe Asp Leu Gly Gly Leu  
1 5 10 15  
Leu His Gly  
20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Glu Leu Arg His Arg Leu Gly Arg Ala Leu Ser Glu Asp Met Val Arg Gly  
1 5 10 15  
Leu Ala Trp Gly Pro Thr Ser His Cys Ala Thr Val Pro Gly Thr Ser Asp  
20 25 30  
Leu Trp Arg Val Ile Arg Phe Leu  
35 40

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Tyr Ser Phe Val His His Gly Phe Phe Asn Phe Arg Val Ser Trp Arg Glu  
1 5 10 15

- 48 -

Met Leu Ala  
20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gln Val Trp Ser Leu Trp Ala Leu Gly Trp Arg Trp Leu Arg Arg Tyr Gly  
1 5 10 15

Trp Asn Met  
20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Trp Arg Arg Met Glu Leu Asp Ala Glu Ile Arg Trp Val Lys Pro Ile Ser  
1 5 10 15

Pro Leu Glu  
20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Arg Arg Ala Ser Val Cys Gly Pro Leu Leu Ser Lys Arg Gly Tyr Gly  
1 5 10 15

Pro Pro Phe Tyr Leu Ala Gly Met Thr Ala Pro Glu Gly Pro Cys  
20 25 30

- 49 -

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Arg Arg Ala Ser Val Cys Gly Pro Leu His Tyr Trp Gly Leu Gly Gly  
1 5 10 15

Phe Val Asp Leu Trp Gln Glu Thr Thr Gly Val Gly Pro Cys  
20 25 30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GACGGGGCGA TCCTCGTCG 19

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Trp Ala Glu Trp Cys Gly Pro Val Cys Ala His Gly Ser Arg Ser Leu  
1 5 10 15

Thr Leu Leu Thr Lys Tyr His Val Ser Phe Leu Gly Pro Cys Lys Met  
20 25 30

Ile Ala Pro Ile Leu Asp  
35

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- 50 -

(A) LENGTH: 20  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Leu Val Cys Lys Ser Tyr Arg Leu Asp Trp Glu Ala Gly Ala Leu Phe Arg  
1 5 10 15  
Ser Leu Phe  
20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Tyr Arg Trp Gln Gln Gly Val Val Pro Ser Asn Trp Ala Ser Cys Ser Phe  
1 5 10 15  
Arg Cys Gly  
20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ser Ser Phe Ser Leu Trp Leu Leu Met Val Lys Ser Ile Lys Arg Ala Ala  
1 5 10 15  
Trp Glu Leu Gly Pro Ser Ser Ala Trp Asn Thr Ser Gly Trp Ala Ser Leu  
20 25 30  
Ala Asp Phe Tyr  
35

- 51 -

What is claimed is:

Claims

1. A method of determining whether a first protein is capable of physically interacting with a  
5 second protein, comprising:
  - (a) providing a host cell which contains
    - (i) a reporter gene operably linked to a DNA-binding-protein recognition site;
    - (ii) a first fusion gene which expresses a  
10 first fusion protein, said first fusion protein comprising said first protein covalently bonded to a binding moiety which is capable of specifically binding to said DNA-binding-protein recognition site; and
    - (iii) a second fusion gene which expresses a  
15 second fusion protein, said second fusion protein comprising said second protein covalently bonded to a gene activating moiety and being conformationally-constrained; and
  - (b) measuring expression of said reporter gene  
20 as a measure of an interaction between said first and said second proteins.
2. The method of claim 1, wherein said second protein is a short peptide of at least 6 amino acids.
3. The method of claim 1, wherein said second protein is a short peptide is less than or equal to 60  
25 amino acids in length.
4. The method of claim 1, wherein said second protein comprises a randomly generated or intentionally designed peptide sequence.

- 52 -

5. The method of claim 1, wherein said second protein is conformationally-constrained because it is covalently bonded to a conformation-constraining protein.

6. The method of claim 1, wherein said first 5 protein is Cdk2.

7. The method of claim 1, wherein said first protein is Ras or an activated Ras.

8. The method of claim 5, wherein said second protein is embedded within said conformation-constraining 10 protein.

9. The method of claim 5, wherein said conformation-constraining protein is thioredoxin.

10. The method of claim 5, wherein said conformation-constraining protein is a thioredoxin-like 15 molecule.

11. The method of claim 9, wherein said second protein is inserted into the active site loop of said thioredoxin protein.

12. The method of claim 1, wherein said second 20 protein is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of said second protein.

13. The method of claim 1, wherein said host cell is yeast.

25 14. The method of claim 1, wherein said DNA binding domain is LexA.

- 53 -

15. The method of claim 1, wherein said reporter gene is assayed by a color reaction.

16. The method of claim 1, wherein said reporter gene is assayed by cell viability.

5 17. A method of detecting an interacting protein in a population of proteins, comprising:

(a) providing a host cell which contains

(i) a reporter gene operably linked to a DNA-binding-protein recognition site; and

10 (ii) a fusion gene which expresses a fusion protein, said fusion protein comprising a test protein covalently bonded to a binding moiety which is capable of specifically binding to said DNA-binding-protein recognition site;

15 (b) introducing into said host cell a second fusion gene which expresses a second fusion protein, said second fusion protein comprising one of said population of proteins covalently bonded to a gene activating moiety and being conformationally-constrained; and

20 (c) measuring expression of said reporter gene.

18. The method of claim 17, wherein said population of proteins comprises short peptides of between 1 and 60 amino acids in length.

25 19. The method of claim 17, wherein said population of proteins is a set of randomly generated or intentionally designed peptide sequences.

- 54 -

20. The method of claim 17, wherein said population of proteins is conformationally-constrained by covalently bonding to a conformation-constraining protein.

5 21. The method of claim 20, wherein each of said population of proteins is embedded within a conformation-constraining protein.

22. The method of claim 20, wherein said conformation-constraining protein is thioredoxin.

10 23. The method of claim 22, wherein each of said population of proteins is inserted into the active site loop of said thioredoxin.

15 24. The method of claim 17, wherein each of said population of proteins is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of said protein.

25. The method of claim 17, wherein said first protein is Cdk2.

20 26. The method of claim 17, wherein said first protein is Ras or an activated Ras.

27. The method of claim 17, wherein said host cell is yeast.

28. The method of claim 17, wherein said DNA binding domain is LexA.

25 29. The method of claim 17, wherein said reporter gene is assayed by a color reaction.

- 55 -

30. The method of claim 17, wherein said reporter gene is assayed by cell viability.

31. A method of identifying a candidate interactor, comprising:

5 (a) providing a reporter gene operably linked to a DNA-binding-protein recognition site;

(b) providing a first fusion protein, said first fusion protein comprising a first protein covalently bonded to a binding moiety which is capable of

10 specifically binding to said DNA-binding-protein recognition site;

(c) providing a second fusion protein, said second fusion protein comprising a second protein covalently bonded to a gene activating moiety and being

15 conformationally-constrained, said second protein being capable of interacting with said first protein;

(d) contacting said candidate interactor with said first protein and/or said second protein; and

(e) measuring expression of said reporter gene.

20 32. The method of claim 31, wherein providing said first fusion protein comprises providing a first fusion gene which expresses said first fusion protein and wherein providing said second fusion protein comprises providing a second fusion gene which expresses said 25 second fusion protein.

33. The method of claim 31, wherein said first fusion protein and said second fusion protein are permitted to interact prior to contact with said candidate interactor.

- 56 -

34. The method of claim 31, wherein said first fusion protein and said candidate interactor are permitted to interact prior to contact with said second fusion protein.

5 35. The method of claim 31, wherein said candidate interactor is conformationally-constrained.

36. The method of claim 31, wherein said candidate interactor is an antagonist and reduces reporter gene expression.

10 37. The method of claim 31, wherein said candidate interactor is an agonist and increases reporter gene expression.

15 38. The method of claim 31, wherein said candidate interactor is a member selected from the group consisting of proteins, polynucleotides, and small molecules.

39. The method of claim 31, wherein said candidate interactor is encoded by a member of a cDNA or synthetic DNA library.

20 40. The method of claim 31, wherein said candidate interactor is a mutated form of said first fusion protein or said second fusion protein.

25 41. The method of claim 32, wherein said reporter gene, said first fusion gene, and said second fusion gene are included on a single piece of DNA.

42. The method of claim 31, wherein said first protein is Cdk2.

- 57 -

43. The method of claim 31, wherein said first protein is Ras or an activated Ras.

44. A population of eukaryotic cells, each cell having a recombinant DNA molecule encoding a 5 conformationally-constrained intracellular peptide, there being at least 100 different recombinant molecules in said population, each molecule being in at least one cell of said population.

45. The population of eukaryotic cells of claim 10 44, wherein said intracellular peptide is conformationally-constrained because it is covalently bonded to a conformation-constraining protein.

46. The population of claim 45, wherein said intracellular peptide is embedded within said 15 conformation-constraining protein.

47. The population of eukaryotic cells of claim 45, wherein said conformation-constraining protein is thioredoxin.

48. The population of eukaryotic cells of claim 20 44, wherein said intracellular peptide is conformationally-constrained by disulfide bonds between cysteine residues in the amino-terminus and in the carboxy-terminus of said second protein.

49. The population of eukaryotic cells of claim 25 44, wherein said cells are yeast cells.

- 58 -

50. The population of eukaryotic cells of claim 44, wherein said recombinant DNA molecule further encodes a gene activating moiety covalently bonded to said intracellular peptide.

5 51. The population of eukaryotic cells of claim 44, wherein said intracellular peptide physically interacts with a second recombinant protein inside said eukaryotic cells.

10 52. A method of assaying an interaction between a first protein and a second protein, comprising:

(a) providing a reporter gene operably linked to a DNA-binding-protein recognition site;

15 (b) providing a first fusion protein comprising said first protein covalently bonded to a binding moiety which is capable of specifically binding to said DNA-binding-protein recognition site;

(c) providing a second fusion protein comprising said second protein covalently bonded to a gene activating moiety and being conformationally-constrained;

20 (d) combining said reporter gene, said first fusion protein, and said second fusion protein; and

(e) measuring expression of said reporter gene.

53. The method of claim 52, wherein providing said first fusion protein comprises providing a first fusion gene which expresses said first fusion protein and wherein providing said second fusion protein comprises providing a second fusion gene which expresses said second fusion protein.

30 54. A protein comprising the sequence Leu-Val-Cys-Lys-Ser-Tyr-Arg-Leu-Asp-Trp-Glu-Ala-Gly-Ala-Leu-Phe-Arg-Ser-Leu-Phe (SEQ ID NO: 1).

- 59 -

55. The protein of claim 54, wherein said protein is conformationally-constrained.

56. A protein comprising the sequence Met-Val-  
Val-Ala-Ala-Glu-Ala-Val-Arg-Thr-Val-Leu-Leu-Ala-Asp-Gly-  
5 Gly-Asp-Val-Thr (SEQ ID NO: 2).

57. The protein of claim 56, wherein said protein is conformationally-constrained.

58. A protein comprising the sequence Pro-Asn-  
Trp-Pro-His-Gln-Leu-Arg-Val-Gly-Arg-Val-Leu-Trp-Glu-Arg-  
10 Leu-Ser-Phe-Glu (SEQ ID NO: 3).

59. The protein of claim 58, wherein said protein is conformationally-constrained.

60. A protein comprising the sequence Ser-Val-  
Arg-Met-Arg-Tyr-Gly-Ile-Asp-Ala-Phe-Phe-Asp-Leu-Gly-Gly-  
15 Leu-Leu-His-Gly (SEQ ID NO: 9).

61. The protein of claim 60, wherein said protein is conformationally-constrained.

62. A protein comprising the sequence Glu-Leu-  
Arg-His-Arg-Leu-Gly-Arg-Ala-Leu-Ser-Glu-Asp-Met-Val-Arg-  
20 Gly-Leu-Ala-Trp-Gly-Pro-Thr-Ser-His-Cys-Ala-Thr-Val-Pro-  
Gly-Thr-Ser-Asp-Leu-Trp-Arg-Val-Ile-Arg-Phe-Leu (SEQ ID  
NO: 10).

63. The protein of claim 62, wherein said protein is conformationally-constrained.

- 60 -

64. A protein comprising the sequence Tyr-Ser-Phe-Val-His-His-Gly-Phe-Phe-Asn-Phe-Arg-Val-Ser-Trp-Arg-Glu-Met-Leu-Ala (SEQ ID NO: 11).

5 65. The protein of claim 64, wherein said protein is conformationally-constrained.

66. A protein comprising the sequence Gln-Val-Trp-Ser-Leu-Trp-Ala-Leu-Gly-Trp-Arg-Trp-Leu-Arg-Arg-Tyr-Gly-Trp-Asn-Met (SEQ ID NO: 12).

10 67. The protein of claim 66, wherein said protein is conformationally-constrained.

68. A protein comprising the sequence Trp-Arg-Arg-Met-Glu-Leu-Asp-Ala-Glu-Ile-Arg-Trp-Val-Lys-Pro-Ile-Ser-Pro-Leu-Glu (SEQ ID NO: 13).

15 69. The protein of claim 68, wherein said protein is conformationally-constrained.

70. A protein comprising the sequence Trp-Ala-Glu-Trp-Cys-Gly-Pro-Val-Cys-Ala-His-Gly-Ser-Arg-Ser-Leu-Thr-Leu-Leu-Thr-Lys-Tyr-His-Val-Ser-Phe-Leu-Gly-Pro-Cys-Lys-Met-Ile-Ala-Pro-Ile-Leu-Asp (SEQ ID NO: 17).

20 71. The protein of claim 70, wherein said protein is conformationally-constrained.

72. A protein comprising the sequence Leu-Val-Cys-Lys-Ser-Tyr-Arg-Leu-Asp-Trp-Glu-Ala-Gly-Ala-Leu-Phe-Arg-Ser-Leu-Phe (SEQ ID NO: 18).

25 73. The protein of claim 72, wherein said protein is conformationally-constrained.

- 61 -

74. A protein comprising the sequence Tyr-Arg-Trp-Gln-Gln-Gly-Val-Val-Pro-Ser-Asn-Trp-Ala-Ser-Cys-Ser-Phe-Arg-Cys-Gly (SEQ ID NO: 19).

75. The protein of claim 74, wherein said protein  
5 is conformationally-constrained.

76. A protein comprising the sequence Ser-Ser-Phe-Ser-Leu-Trp-Leu-Leu-Met-Val-Lys-Ser-Ile-Lys-Arg-Ala-Ala-Trp-Glu-Leu-Gly-Pro-Ser-Ser-Ala-Trp-Asn-Thr-Ser-Gly-Trp-Ala-Ser-Leu-Ala-Asp-Phe-Tyr (SEQ ID NO: 20).

10 77. The protein of claim 76, wherein said protein  
is conformationally-constrained.

78. Substantially pure DNA encoding the protein  
of claim 54.

15 79. Substantially pure DNA encoding the protein  
of claim 56.

80. Substantially pure DNA encoding the protein  
of claim 58.

81. Substantially pure DNA encoding the protein  
of claim 60.

20 82. Substantially pure DNA encoding the protein  
of claim 62.

83. Substantially pure DNA encoding the protein  
of claim 64.

25 84. Substantially pure DNA encoding the protein  
of claim 66.

- 62 -

85. Substantially pure DNA encoding the protein of claim 68.

86. Substantially pure DNA encoding the protein of claim 70.

5 87. Substantially pure DNA encoding the protein of claim 72.

88. Substantially pure DNA encoding the protein of claim 74.

10 89. Substantially pure DNA encoding the protein of claim 76.

90. A protein isolated by a method comprising:

(a) providing a host cell which contains

(i) a reporter gene operably linked to a DNA-binding-protein recognition site; and

15 (ii) a fusion gene which expresses a fusion protein, said fusion protein comprising a test protein covalently bonded to a binding moiety which is capable of specifically binding to said DNA-binding-protein recognition site;

20 (b) introducing into said host cell a second fusion gene which expresses a second fusion protein, said second fusion protein comprising one of said population of proteins covalently bonded to a gene activating moiety and being conformationally-constrained; and

25 (c) measuring expression of said reporter gene; and

(d) isolating a protein based on its ability to alter the expression of said reporter gene when present in said second fusion protein.

- 63 -

91. An interactor protein isolated by a method comprising:

(a) providing a reporter gene operably linked to a DNA-binding-protein recognition site;

5 (b) providing a first fusion protein, said first fusion protein comprising a first protein covalently bonded to a binding moiety which is capable of specifically binding to said DNA-binding-protein recognition site;

10 (c) providing a second fusion protein, said second fusion protein comprising a second protein covalently bonded to a gene activating moiety and being conformationally-constrained, said second protein being capable of interacting with said first protein;

15 (d) contacting a candidate interactor protein with said first protein or said second protein;

(e) measuring expression of said reporter gene; and

20 (f) isolating an interactor protein based on its ability to alter the expression of said reporter gene when present with said first protein or said second protein.

1 / 7

FIG. 1A

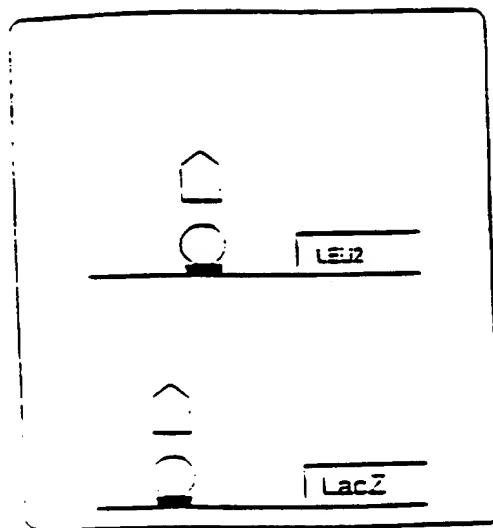


FIG. 1B

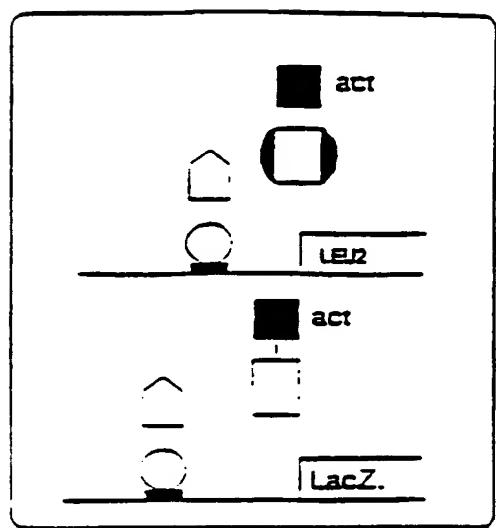
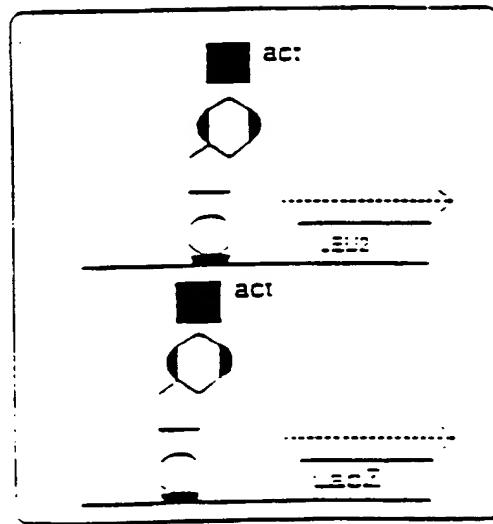


FIG. 1C



2 / 7

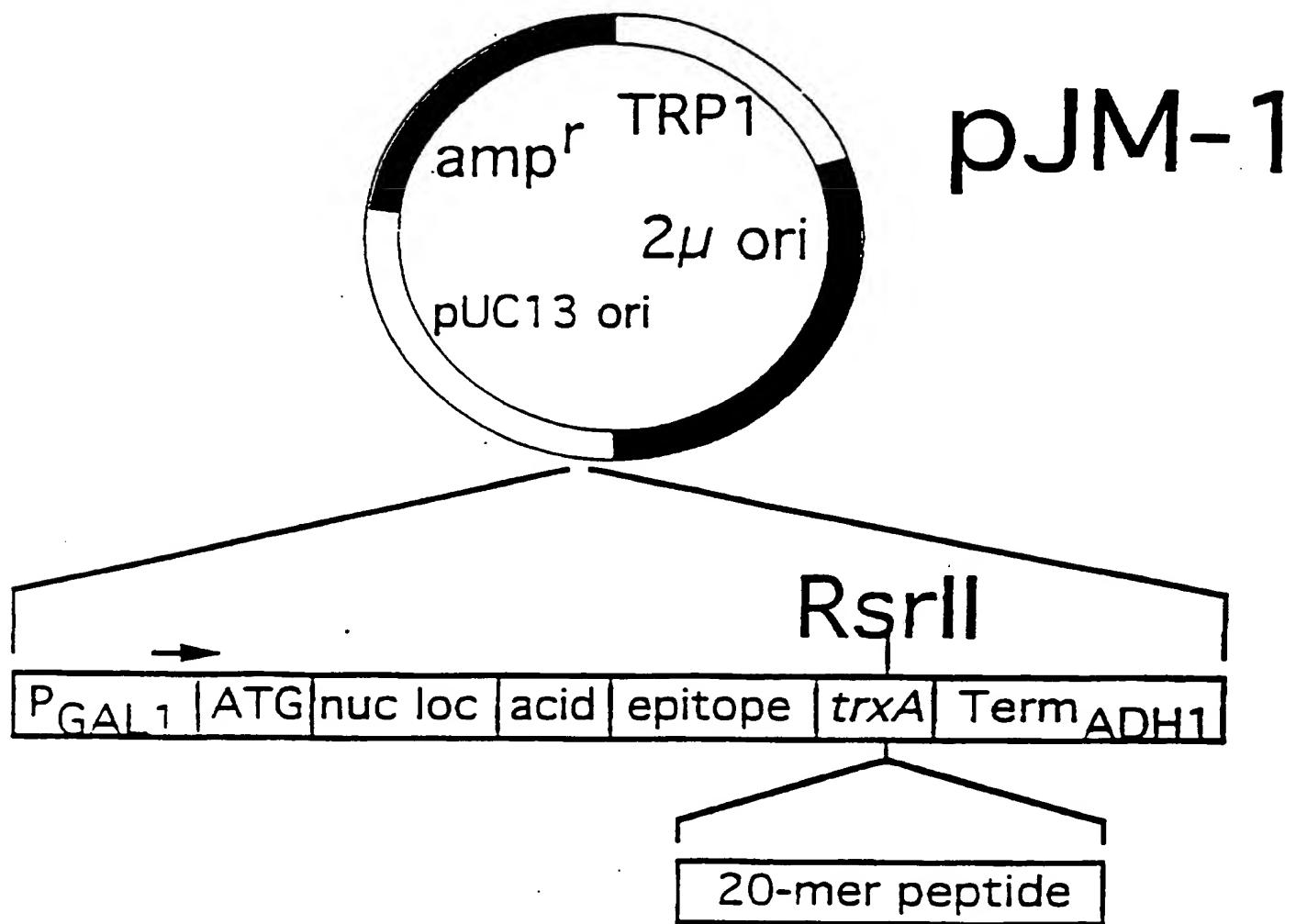
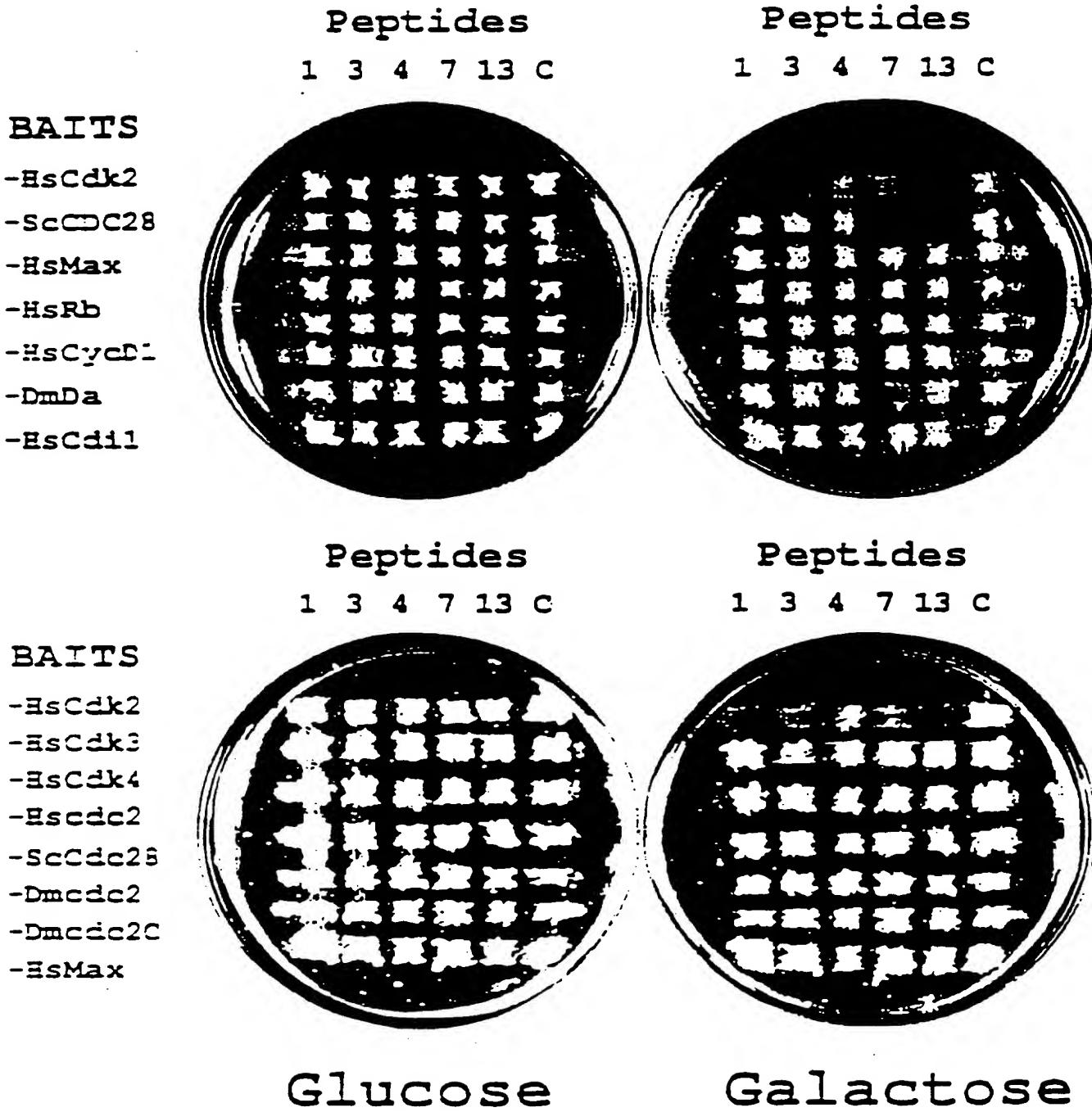


FIGURE 2

3 / 7

FIGURE 3A



4 / 7

**FIGURE 3B**

**Peptide 13: SVRMRYGIDAFFDLGGLLHG (SEQ ID NO:9)**

**Peptide 1: ELRHRLGRAL SEDMVVRGLAW GPTSHCATVPG TSDLWRVIRFL (SEQ ID NO:10)**

**Peptide i5-1: YSFVHHGFFNFRVSWREMLA (SEQ ID NO:11)**

**Peptide i5-4: QVWSLWALGWRWLRRYGNM (SEQ ID NO:12)**

**Peptide i5-9: WRRMELDAEIRWVKPISPLE (SEQ ID NO:13)**

**Peptide 3: LVCKSYRLDW EAGALFRSLF (SEQ ID NO:18)**

**Peptide 4: YRWQQGVVPS NWASCSFRCCG (SEQ ID NO:19)**

**Peptide 7: SSFSLWLLMV KSIKRAAWEL GPSSAWNTSG WASLADFY (SEQ ID NO:20)**

5 / 7

**Figure 4**

GST	GST-CDK2	
Trx	3	13

6 / 7

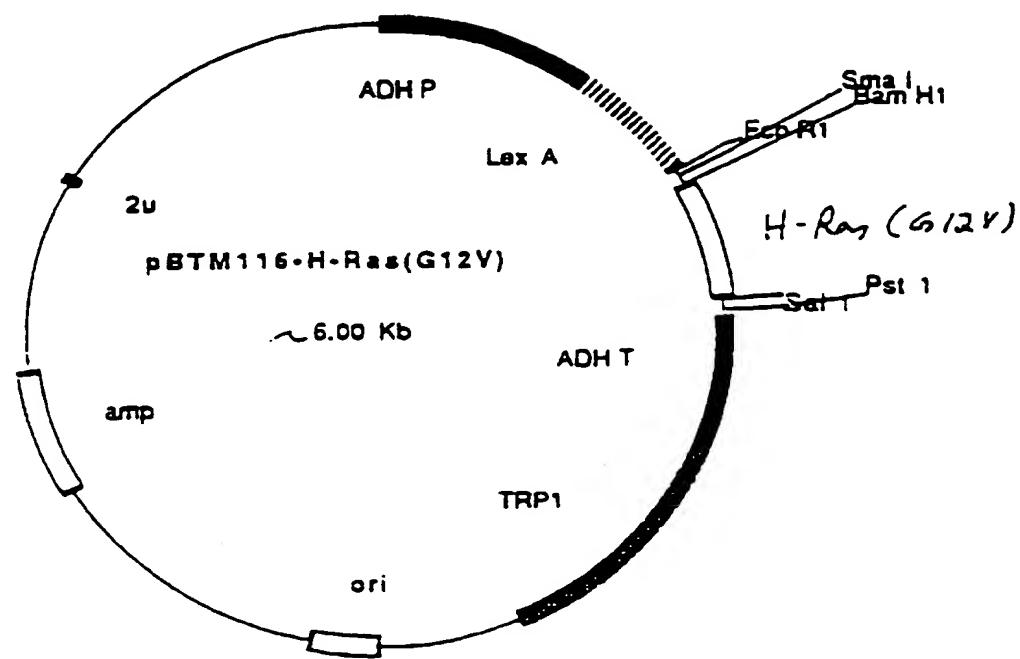


Figure 5

7 / 7

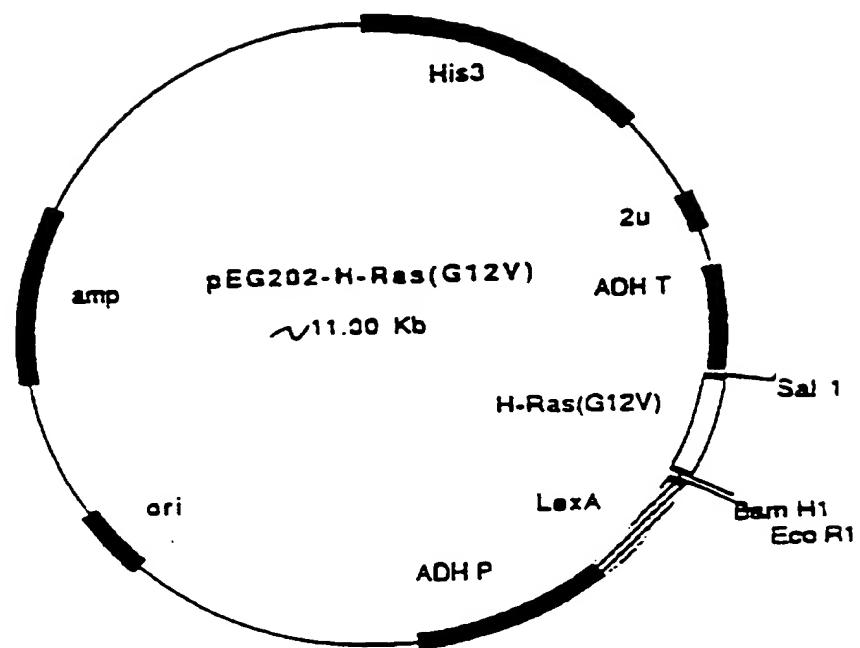


Figure 6

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/09307

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C07K 19/00; C12N 1/19, 5/10; C12Q 1/68; G01N 33/68

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.2, 7.21, 7.31, 240.2, 243, 254.21; 530/324, 326, 350; 536/23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	Trends in Genetics, Vol. 10, No. 8, issued August 1994, Fields et al, "The Two-Hybrid System: an Assay for Protein-Protein Interactions", pages 286-292, see entire document.	1-53, 90-91
X ---	US, A, 5,283,173 (FIELDS ET AL) 01 February 1994, see entire document.	1-5, 13, 15-20, 27, 29-30, 44, 49-53, 90
Y		----- 6-12, 14, 21-26, 28, 31-43, 45-48, 91

 Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

25 OCTOBER 1995

Date of mailing of the international search report

24 NOV 1995

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/09307

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	D. A. HARTLEY, "Cellular Interactions in Development, A Practical Approach" published 1994 by IRL Press (N.Y.), pages 153-179, see entire document.	1-5, 13-20, 27-30, 44, 49-53, 90 ----- 6-12, 21-26, 31-43, 45-48, 91
X -- Y	Proceedings of the National Academy of Sciences USA, Vol. 88, issued November 1991, Chien et al, "The Two-Hybrid System: A Method to Identify and Clone Genes for Proteins that Interact with a Protein of Interest", pages 9578-9582, especially page 9578-9579.	1, 3, 5, 13, 15-17, 20, 27, 29-30, 44, 49-53, 90 ----- 2, 4, 6-12, 14, 18-19, 21-26, 28, 31-43, 45-48, 91
Y	US, A, 5,270,181 (MCCOY ET AL) 14 December 1993, see columns 4-5, 10, and 12-16.	1-53, 90-91
Y	Journal of Biological Chemistry, Vol. 267, No. 25, issued 05 September 1992, Chakraborty et al, "Analysis of the Oligomerization of Myogenin and E2A Products <i>in Vivo</i> using a Two-Hybrid Assay System", pages 17498-17501, especially page 17500, paragraphs 2-3.	31-43, 91
Y	Cell, Vol. 75, issued 19 November 1993, Harper et al, "The p21 Cdk-Interacting Protein Cip1 is a Potent Inhibitor of G1 Cyclin-Dependent Kinases", pages 805-816, especially pages 805 and 806, column 2.	6, 25, 42

**INTERNATIONAL SEARCH REPORT**

International application No. PCT/US95/09307
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**A. CLASSIFICATION OF SUBJECT MATTER:**

US CL :

435/6, 7.1, 7.2, 7.21, 7.31, 240.2, 243, 254.21; 530/324, 326, 350; 536/23.4

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

USPAT, MEDLINE, BIOSIS, SCISEARCH, MPsearch\_pp  
search terms: interaction trap, two-hybrid system, protein#, protein-protein, peptide#, thioredoxin, DNA bind###,  
hybrid#, GAL4, LEXA, Fields S, Song O, Brent, R

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